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以斑馬魚為模式研究神經內分泌的新功能

Zebrafish an outstanding molecular model system to study novel physiological function of neuroendocrine related hormones

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本論文係柯雪莉君（D94B45002）在國立臺灣大學漁業科學研究所完成之博士學位論文，於民國 100 年 6 月 27 日承下列考試委員審查通過及口試及格，特此證明

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☺

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硬骨魚類在適應環境過程中，鰓以及皮膚上的離子細胞型態與增生深受皮質醇調控。在本研究中探討皮質醇影響離子細胞的背後分子機制，並探討皮質醇受器 (醣皮質醇受器)在魚類表皮離子細胞發育過程中的影響。此外，藉由使用斑馬魚當作模式動物，本實驗提供活體(*in vivo*) 以及體外 (*in vitro*) 的研究分析，同時研究促皮質醇釋放激素、腎上腺素及這些激素相關受器等在皮膚發育過程中的影響。

實驗結果發現，使用皮質醇去刺激斑馬魚受精卵會抑制醣類皮質醇受器基因轉錄，但是不會影響礦物性皮質醇受器基因轉錄。在皮質醇刺激下，斑馬魚的富含鈉鞣浦細胞 (NaRCs) 數目、富含氫鞣浦細胞 (HRCs) 數目、表皮鈣離子通道基因轉錄、*gcm2* 基因轉錄、鈉氫交換蛋白 (*nhe3b*) 基因轉錄以及氫鞣浦 (*atp6v1a*) 基因轉錄等皆會被向上調控。此外，使用原位雜交技術發現離子細胞分化指標基因 *foxi3a* 跟 *foxi3b* 的 mRNA 表現會在皮質醇處理 24 到 48 小時後被刺激。但是在細胞分裂分析上則沒觀察到有被影響。在細胞凋亡的分析上則發現皮質醇在不同發育時間會有不同影響，在受精後 48 小時是減少但是受精後 72 小時是增加。

使用注射反義核苷酸剔除技術抑制醣類皮質醇受器的蛋白質轉譯，結果則發現醣類皮質醇受器的減少會降低富含鈉鞣浦離子細胞以及富含氫鞣浦離子細胞的數目。

相反的，抑制礦物性皮質醇受器的蛋白質轉譯則不會影響表皮幹細胞跟離子細胞數目。在體外培養斑馬魚鰓的實驗上則發現，處理 24 小時的皮質醇會刺激離子細胞數目跟分化指標基因 foxi3a/b 的轉錄。因此我們推論皮質醇是經由醣類皮質醇受器來調控 foxi3a/b 基因轉錄，進而影響前驅離子細胞的的特化與分化，並且皮質醇能延遲離子細胞凋亡。這樣的皮質醇作用最後促使成熟離子細胞的數目明顯增生。

章節二：促皮質醇釋放因子及其受器或相關蛋白質在斑馬魚皮膚發遇上的影響

下視丘-腦下垂體-間腎組織間軸 (HPI axis) 等系統性影響已經被證實在皮膚發育上扮演重要的角色。使用注射反義核苷酸技術抑制促皮質醇釋放因子及其第一型受器(CRFR1)蛋白質轉譯，發現會明顯影響表皮幹細胞跟離子細胞密度。CRFR1 主要功能在於調節促皮質醇釋放因子功能，並且發現該受器在斑馬魚卵一開始受精後就存在。促皮質醇釋放因子第二型受器 (CRFR2)則未被發現能夠影響表皮幹細胞跟離子細胞密度。儘管如此，CRFR2 在其他生理功能上的重要性仍不能夠被排除。

目前研究證實 HPI axis 能以系統性或是局部性的作用影響斑馬魚皮膚跟鰓的功能。促皮質醇釋放因子能夠以 CRFR1 來直接影響表皮幹細胞進而調控離子細胞分化，此外促皮質醇釋放因子也能夠經由影響皮質醇分泌，進而調控 *foxi3a/b* 基因轉錄來達到相

同功能。未來研究需要再詳細去闡明神經內分泌系統在調控表皮離子細胞分化上的機制。

第二部分

章節一：斑馬魚飢餓激素 (Ghrelin)經由抑制腦中的胰島素或刺激昇糖激素(glucagon)來影響腦中糖類碳水化合物的代謝

糖類碳水化合物的代謝所產生能量對於中樞神經系統的能量供給非常重要。在胰臟，飢餓激素已被知道對於糖類碳水化合物代謝跟調控胰島素扮演非常明顯重要的角色。然而在除了胰臟的其他組織上，飢餓激素對於胰島素調控仍然不清楚。在這研究，我們使用斑馬魚成魚來探討斑馬魚飢餓激素在腦中的表現跟影響糖類碳水化合物代謝的功能。根據 RT-PCR 跟原位雜交技術的結果顯示：斑馬魚腦會表現飢餓激素與其受器 (GHS-R: *zghs-r1a* and *zghs-r2a*) 。 在蛋白質定位的證據更確認斑馬魚腦表現飢餓激素的事實。當注射合成的金魚飢餓激素 peptide 到斑馬魚成魚，結果發現會刺激斑馬魚腦中的 *zghs-r1a* 跟 *zghs-r2a* 轉錄，進一步分析發現，魚腦中的胰島素跟其受器會被明顯抑制，但是昇糖激素跟其受器則會被明顯刺激，在糖類碳水化合物代謝後，緊接而至的急迫肝醣感受反應將會平衡肝醣降解跟能量儲存。這些發現推論：在中樞神經系統，飢餓激素藉由調節其受器來控制胰島素合成，並且影響糖類碳水化合物代謝。

BACKGROUND OF THE STUDY

Extensive studies have been exerted to support and augment the need of a sustainable fish industry around the globe. Hence, more research venues on fisheries science continuously expand and slowly taking part to answer questions related to biomedical related issues that indeed remain essential to fish.

Zebrafish is highly regarded as one of the top model organisms where mouse and rat were commonly known for (McGonnell and Fowkes, 2006; Löhr and Hammerschmidt, 2011). Highly utilized as a model system, many fields of research prefer to use zebrafish, for both forward (identifying the genetic basis of a phenotype) and reverse (analyzing phenotypic effect of a gene) genetics (Abraham et al., 2010; Lieschke and Currie, 2007; Rubinstein, 2003, 2006; Zon, 2005). The high fecundity of zebrafish, fast and complete development within a day, and transparency at early developmental stages are qualities highly ideal for molecular assays. The zebrafish genome is available and almost complete and highly accurate. In the present study, we took advantage of this model system and the availability of new molecular techniques to do further research in the endocrine system focusing on skin development and endocrine energy metabolism related function.

The present study focused on classical (corticotropin releasing factor and related proteins, cortisol and associated receptors, insulin and glucagon) and newly discovered neuroendocrine peptides (ghrelin and receptors), which are known to be multifunctional, and yet discovering their novel physiological functions will essentially contribute additional knowledge in fisheries science.

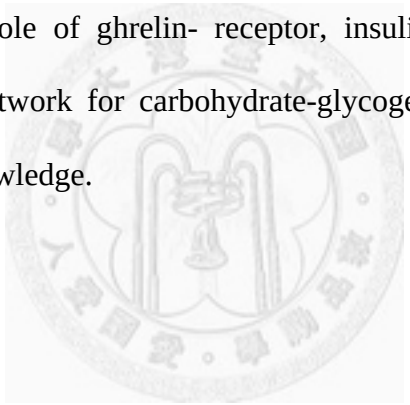
PURPOSE OF THE STUDY

Using zebrafish as a model organism, novel functions of cortisol–*glucocorticoid/mineralocorticoid receptor* axis and *corticotrophin releasing factor-corticotropin releasing factor receptor 1/2* and *melanocorticotropin 2 receptor* roles in skin development including *ghrelin* and related metabolic factors insulin and glucagon were delineated.

1. **PART I (Chapter I)** Cortisol is the main endogenous glucocorticoid (GC) in fish and human, unlike in rodents which are corticosterone (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997; Schaaf *et al.*, 2009). Together with the extensive studies in mammalian and teleost cortisol-GR effect in skin physiological processes, this study will clarify and further understand the underlying molecular mechanism involved in skin development. In addition of the existing epidermal ionocyte development proposed molecular mechanism, facilitated by *foxi3a/3b* regulatory loop and the demarcated developmental stages (Hsiao *et al.*, 2007; Janicke *et al.*, 2007) the present study utilizes this knowledge and extends its connection to endocrine factors discovering their novel function. The molecular mechanism of cortisol—GR action in zebrafish epidermal ionocytes development will be elucidated by cortisol treatment, loss of function assay and organ culture.

2. **PART (Chapter II)** In the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, CRF plays a major contribution and initializes this event from the central nervous system (CNS) to the peripheral tissues (Turnbull and Rivier, 1997). Expression of CRF in fish gills is very much documented to all species studied, signifying a primary role of CRF to the major physiological activity of fish such as osmo-/ionoregulation. CRF was known to be produced in the skin and from skin neural ends (Slominski and Wortsman, 2000). In addition, the skin neuroendocrine system functions to preserve and maintain cutaneous structural and functional integrity and systemic homeostasis (Slominski and Wortsman, 2000). Therefore, to elucidate the role of CRF in skin development bearing in mind its major physiological importance in the survival of fish, this study is an essential contribution to clarify its worth. Using zebrafish as the model system to study local signaling pathway of CRF and related proteins down to cortisol as mentioned above (PART I- Chapter I) may open new avenues for research and better understanding in the skin development of teleost fish.

3. **PART II (Chapter I)** Ghrelin is the only peptide known to date that could highly stimulate the release of GH (Bowers *et al.,* 1977; Bowers *et al.,* 1980). Newly discovered neuropeptide ghrelin (Kojima et al., 1999) contributes to a wide array of biological functions; however, a role in energy metabolism and the vast cascade of downstream mechanisms has not been clearly delineated. In the brain, the high demand of energy is efficiently met by glucose supply; insulin and glucagon plays the major role in this process. Ghrelin is associated to insulin and glucagon as inhibitory or stimulatory signal; a controversial issue needs to be settled so far. The possible link between ghrelin and these metabolic-related proteins may provide a better understanding on the maintenance of the energy supply and energy storage in the brain. Exploring the role of ghrelin- receptor, insulin and glucagon in the CNS complex regulatory network for carbohydrate-glycogen metabolism is an essential contribution to our knowledge.



PART I

Chapter I

Cortisol controls epidermal ionocyte differentiation and proliferation by targeting Foxi3 transcription factors in zebrafish (*Danio rerio*)

ABSTRACT

In teleost fish, skin/gill function, cell morphological changes and cell proliferation were greatly affected by cortisol during environmental acclimation. In the present study we examined the molecular mechanism behind cortisol action including its known receptor, *glucocorticoid receptor* (*gr)* in fish epidermal ionocyte progenitor development. Utilizing zebrafish for both *in vivo* and *in vitro* assay, together with the involvement of the neuroendocrine system in the skin development which includes the *corticotrophin releasing factor* (*crf)*, *crf receptor 1* (*crfr1*), *crfr2* and *melanocotropin 2 receptor* (*mc2r*) or *ACTH receptor*.

Cortisol treatment of zebrafish newly fertilized eggs suppressed *gr* transcripts without affecting *mr*. Transcripts of ionocyte marker genes for Na^+-K^+-ATPase rich cells (NaRCs): *epithelial* Ca^+ *channel* (*ecac*) and H^+-ATPase rich cells (HRCs): *glial cell missing 2* (*gcm2*), *Na^+-H^+-exchanger 3b* (*nhe3b*) (B), H^+-ATPase A-subunit (*atp6v1a*) were all upregulated upon cortisol treatment. Immunocytochemistry confirmed that both NaRCs and HRCs density were significantly increased. In addition, ionocyte progenitor specification and differentiation marker genes *foxi3a and foxi3b* spatial mRNA expression were increased after 24-48 h treatment of cortisol via in situ hybridization. Cell division was not affected but cell apoptosis was decrease at 48 hpf and increased at 72 hpf in the cortisol-treated group.

Knock-down of *gr* by GR-ATG MO showed immense lowered NaRCs and HRCs numbers among GR morphants which is further confirmed by GR-SV MO. In contrast, loss of MR has no effect in epidermal stem cells and ionocyte density. *In vitro*, 24 h gill organ culture with cortisol treatment significantly increased NaRCs and HRCs numbers including

upregulation of *foxi3a/b* transcripts. Hence, we propose that cortisol through *gr* targets *foxi3a/b* that regulates epidermal ionocyte progenitor specification/differentiation together with delayed apoptosis in which case caused proliferation of matured ionocytes as the apparent outcome.

Introduction

In teleost fish, cortisol contributes a critical role in osmo/ion, metabolic and immune regulation among others (Wedellaar Bonga, 1997; Mommsen *et al.,* 1999; Takahashi *et al,* 2006). Cortisol was tagged as the "seawater-adaptive" response of euryhaline fishes (McCormick, 2001), based on extensive studies of environmental acclimation from freshwater to seawater or vice versa with cortisol exposure. Fish gills ion transporters enzyme activity (Na^+, K^+-ATPase, NKA), messenger (m)RNA, and protein levels (Na^+,K^+,2Cl-cotransporter; α1a and α1b subunits of NKA) were stimulated by exogenous cortisol in rainbow trout, *Salmo gairdneri* (Perry and Laurent, 1989); freshwater coho salmon, *Oncorhynchus kisuth* (McCormick and Bern, 1989, Yada *et al.,* 2008); freshwater rainbow trout, *Oncorhynchus mykiss* (Shrimpton and McCormick, 1999); brown trout, *Salmo trutta*, and Atlantic salmon, *Salmo salar* (Tipsmark *et al*., 2002; Kiilerich *et al.,* 2007a; Kiilerich *et al.,* 2011); seawater silver sea bream, *Sparus sarba* (Deane and Woo, 2005); and Japanese eel, *Anguilla japonica* (Wong and Chan, 1999). All these were accompanied by skin/gill chloride cell morphological changes and increase in number. For instance, in tilapia (*Oreochromis mossambicus)*, cortisol treatment increased chloride cell density in a dose-dependent manner (in vitro), and suggested that cortisol regulates differentiation of chloride cells (McCormick, 1990). Upon discovery of cortisol receptor in branchial chloride cell and undifferentiated cells of chum salmon fry (*Oncorhyncus keta*); cortisol was implicated in the functional differentiation of chloride cell (Uchida *et al*., 1998). Transitory characteristic of chloride cells in eel (*Anguilla japonica*) was shown upon freshwater transfer to seawater and suggested that observed mitotic cell believed to be stem cells produces undifferentiated cells that would eventually become chloride cells (Wong and Chan, 1999). Similarly, in tilapia (*Oreochromis mossambicus*), chloride cell developmental stages was classified and proposed that the sequential morphological change from freshwater-type to seawater-type reflects

plasticity of chloride cell ion-transport function (Hiroi *et al.,* 1999). In addition, cortisol implantation in rainbow trout increased gills number of Na^+/K^+-ATPase-rich cells (Shahsavarani and Perry, 2006). As described above, conclusive ideas of cortisol inducing gill cells morphological changes is "cell differentiation" and increase in numbers as evidence of "cell proliferation" based on biochemical analysis, flow cytometry and other histo/cytological analysis (Perry and Laurent, 1989; McCormick, 1990; Laurent *et al*., 1994; Hiroi *et al.,* 1999; Wong and Chan, 1999; Wong and Chan, 2001; Sloman *et al.,* 2001; Scott *et al.,* 2005). During this so-called "cell development", the question lies on what is the exact target of cortisol action in the process of epidermal ionocyte (EI) development, since not long ago that specific demarcation of time and genes involved during EI development was laid-out in teleost fish (Jänicke *et al*., 2007; Hsiao *et al.,* 2007).

Likewise, GC is highly regarded hormone in mammalian studies and established to be a potent inhibitor of epidermal proliferation, hence, it is widely used for hyperproliferative epithelial problem and many human skin disorders (Verma *et al.,* 1983; Ahluwalia, 1998; Schäcke *et al.,* 2002). Similarity of glucocorticoid which is cortisol in both human and fish signifies a similar physiological response such as in stress axis as suggested previously (Chandeskar *et al.,* 2007). Tissue-specific study using primary human keratinocytes demonstrates dexamethasone, a GC analog, promotes transcriptional regulation of genes related to cell fate, differentiation, and metabolism using microarray analysis (Stojadinovic *et al.,* 2007). In vitro, human keratinocyte proliferation was inhibited within 48-72 h GC treatment, but promoted transcript expressions of genes related to keratinocyte terminal differentiation within 24, 48, 72 h GC treatment (Stojadinovic *et al.,* 2007). On the other hand, mouse devoid of glucocorticoid receptor ($GR^{-/-}$), skin barrier competence was compromised with incomplete epidermal stratification and dramatic loss of terminal differentiation (Bayo *et al.,* 2008). This absence of keratinocyte terminal differentiation was a

result of impaired activation of nonapoptotic caspase 14 (Bayo *et al.,* 2008), a requirement for keratinocyte differentiation (Fisher et al., 2005). In addition, cultured primary keratinocytes from $GR^{-/-}$ mice demonstrated increased growth/proliferation and cell apoptosis in cell-autonomous manner (Bayo *et al.,* 2008). Whereas transgenic mice overexpressing GR under the control of keratin K5 promoter gene (K5-GR mice) developed immense skin defects, such as epidermal hypoplasia and some exhibits complete absence of all epidermal layers among others (Pérez *et al.*, 2001); induces hypohidrotic ectodermal dysplasia, a human syndrome defined by maldevelopment of one or more ectodermal-derived tissues that includes epidermis and cutaneous appendices (Cascallana *et al.,* 2005). Moreover, the offspring of cross-bred mice male (K5-GR mice) with female that contains v-Ha-ras oncogene inducing carcinogenesis (Tg.AC mice) demonstrates dramatic inhibited skin tumor development (Budunova *et al.,* 2003). Collectively, mammalian along with teleost extensive knowledge of cortisol-GR effect in skin physiological processes paved the way to clarify and further understand the underlying molecular mechanism involved in skin development.

Availability of molecular evidence recently emerged to demonstrate the foundation of teleost epidermal ionocyte development using zebrafish as a premier model in molecular studies. A homolog of the transcription factor p53, p63 was found required in nonneural ectoderm (epidermal cells) regenerative characteristics (Mills *et al.,* 1999; Yang and Mckeon, 2000; Bakkers *et al.,* 2002). Consequently, p63 became as the epidermal stem cell marker followed by rigorous study on skin epidermal developmental process. The master regulator in zebrafish EI specification and differentiation has been established as carried-out by *foxi3a-foxi3b* regulatory loop, duplicated winged helix/forkhead transcription factors (Ezaki *et al.,* 2009; Jänicke *et al.,* 2007; Hsiao *et al.,* 2007; Solomon *et al.*, 2003). In addition, zebrafish transcription factor glial cell missing 2 (*gcm2*) was shown involved in the differentiation and functional regulation of an ionocyte cell-type H^+-ATPase-rich cells (HRCs) (Chang *et al.,*

2009). There are two specific types of ionocytes that develops in the early life of zebrafish that can be observed in the skin and developing gills, the Na^+-K^+-ATPase-rich cells (NaRCs) and (HRCs) (Lin *et al.,* 2006). The involvement of *foxi3a/b* and *gcm2* as possible target genes of cortisol-GR signaling pathway in EI development is the main concern of this study. Noteworthy, GR is known for its transcriptional regulatory effect with variety of transcription factors (Zhou and Cidlowski, 2005). Whether cortisol affects epidermal stem cell growth/proliferation, ionocyte progenitor specification/differentiation, maturation and terminal differentiation (apoptosis) are issues need to be settled.

GC is traditionally known mediated by GR, a well-known transcription factor (Beato *et al.*, 1995). Inactive GR resides in the cytosol associated with heat-shock proteins complex; upon ligand binding, GR dissociates from the complex and translocates to the nucleus as homodimer to specific DNA motifs called glucocorticoid-response elements (GREs) and activates transcription of target genes (Beato *et al.,* 1995; Ismaili and Garabedian, 2004). GR is a member of the nuclear receptor (NR) superfamily that belongs to the steroid receptor subfamily. Most teleostean fishes possessed two GR genes, while zebrafish genome contains only one GR gene that produces two spliced variants (Schaaf *et al.,* 2008; Alsop and Vijayan, 2008). Similarly, MR belongs also to NR superfamily. This relatedness with GR apparently reflects similar physiological functions between GR and MR (Pascual-le Tallec and Lombes, 2005). Since fish do not possess aldosterone (Jiang *et al.,* 1998), a hormone responsible for the reabsorption of sodium and the release/secretion of potassium in the kidneys, cortisol was suggested to compensate for the role through MR (Prunet *et al.,* 2006; Bury *et al.,* 2003). For instance, cortisol was shown to regulate ion uptake and salt secretion in Atlantic salmon, evidence of mineralocorticoid role (McCormick *et al.,* 2008). After the rainbow trout mineralocorticoid-like receptor cloning and characterization (Colombe *et al.,* 2000), followed reports on the blockage of this receptor by spironolactone an antagonist with high affinity to

mammalian MR. In rainbow trout, spironolactone inhibited branchial chloride cell proliferation (Sloman *et al.,* 2001), while both cell proliferation and expression of osmoregulatory related enzymes were inhibited in killifish (Scott et al., 2005) in environmental acclimation experiments and were suggested to be an evidence of mineralocorticoid action by cortisol. In contrary, McCormick *et al.*, (2008) demonstrated that using the similar antagonist spironolactone did not elicit any effect on NKA activity and expression level of NKA subunits, α1a and α1b. On the other hand, 11-deoxycorticosterone (DOC), a precursor molecule for the production of aldosterone, is present in fish (Jiang *et al.,* 1998), and was shown to be a potent agonist of MR in rainbow trout (*Oncorhynchus mykiss*) (Sturm *et al.,* 2005). Hence, DOC is a possible ligand for MR in fish. Nevertheless, whether GR or MR participates in mediating cortisol action needs clarification, and cortisol dual role could not be disregarded.

Programmed cell death is part of the whole process of dynamic equilibrium in skin epidermal development with continuous replenishment. In vertebrates, the epidermis constant state of renewing itself is possible by multipotent epithelial stem cells (Oshima *et al.*, 2001). These stem cells divide and produce a daughter/progenitor cell that eventually differentiates. Once terminally differentiated this cell reach the body surface and consequently undergo programmed cell death and is continually replenished (Fuchs and Raghavan, 2002). A potent synthetic cortisol analogue dexamethasone was shown to block UV-induced apoptosis in primary human keratinocytes (Stojadinovic *et al.,* 2007). In addition, cultured mouse primary keratinocytes from $GR^{-/-}$ knockout mice demonstrate increased apoptosis (Bayo *et al.,* 2008). In contrast, glucocorticoid and GR was shown to stimulate apoptosis in several epidermal cell types in mammalian studies (Distelhorst, 2002; Budunova *et al.,* 2003). Either cortisol stimulates or inhibits EI apoptosis in teleost fish is not completely settled.

Altogether, the present study will do its best to elucidate the cortisol action in the teleost fish skin development since very scarce molecular evidence has been provided so far. This study aims to clarify the following specific issues: (1) cortisol's action in zebrafish skin development specifically epidermal progenitor specification, differentiation and proliferation, (2) to demonstrate the molecular mechanism involved in the skin development through *foxi3a/3a* regulatory role in relation to cortisol mediated action, (3) to identify the role of cortisol associated receptors (*gr* and *mr*) in mediating its action, and lastly, (4) to extend the scope of the study by checking cortisol up-stream signals involved in the HPI axis and their role in skin development.

MATERIALS AND METHODS

Experimental animals

Adult zebrafish (*Danio rerio*) were obtained from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. Fish were reared in a circulating system containing local fresh water at 27~28 C under a 14: 10 h light: dark photoperiod. Daily feeding consisted of artificial feed pellets (Fu-So, Taipei, Taiwan). The Academia Sinica Institutional Animal Care and Utilization Committee approved all the experiment protocols used in this study.

Cortisol treatment of newly fertilized zebrafish eggs

Previous cortisol experiment in tilapia embryo (Lin *et al.*, 1999) showed that 10mg/L of cortisol effectively stimulated Na^+ body content after 4-8h transfer to seawater. In the case of zebrafish cortisol treatment from our team (unpublished data), we tested several hydrocortisone-21-hemisuccinate (Sigma) concentrations such as 10, 20, and 40 mg/L. From this preliminary experiment dosage of 20 mg/L showed to be the most suited level to adequately stimulate *gr* mRNA level as well as other enzymes involved in the cortisol synthesis, hence, we decided to choose 20mg/L for all succeeding experiments. Newly fertilized zebrafish eggs are grown in normal tap water with 20 mg/L hydrocortisone-21-hemisuccinate and incubated in controlled system at 28 C together with the control group (tap water only). Time-course collections of samples are designated and ionocytes are quantified by immunocytochemistry analysis using specific antibodies to each type of ionocyte (NaRCs and HRCs) including epidermal stem cells.

Total RNA isolation and first strand (complementary)DNA synthesis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was purified by a MasterPure^{TM} RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). The total amount of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from the purified and quantified total RNA using the SuperScript^{TM} kit for reverse-transcription polymerase chain reaction (RT-PCR; Invitrogen).

Reverse-Transcription PCR (RT-PCR)

Total RNA in several developmental stages was isolated from zebrafish embryos: 0 (immediately processed when released from the female zebrafish), 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 36 hours post fertilization (hpf). Similar to different tissue samples, the following were collected: brain, eyes, gills, heart, intestine, liver, spleen, intestine, kidney, skin and muscle. Transcript expressions of the following genes are determined: *gr, mr* and *β-actin* served as the internal positive control. All gene sequences were gathered from NCBI and Ensemble genome databases. All primers used for the whole study were designed using Primer Premier Express software (version 2.0.0; Applied Biosystems). Primers used are listed at TABLE 1. The primer products were amplified by a conventional reverse-transcription polymerase chain reaction (RT–PCR). Analysis was performed with 5 min of denaturation at 95 C, followed by 30 s at 95 C. The annealing time was 30 s at 55~59 C based on the melting temperature of the primers used, and the elongation period was 30~60 s depending on the length of the product at 72 C. All reactions were run for 30 cycles. Amplified transcripts were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA), and all amplicons were sequenced to confirm the desired products.

Quantitative real-time PCR (qPCR)

For the cortisol treatment experiment, mRNA expressions of target genes were measured by qPCR using ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA) and Universal SYBR Green master mix (Applied Biosystems). Primers of target genes were listed in TABLE 2. All qPCR reactions were performed as follows: 1 cycle of 50 C for 2 min and 95 C for 10 min, followed by 40 cycles of 95 C for 15 s and 60 C for 1 min. Control reactions used sterile water to determine background and genomic DNA contamination levels. Values were computed using a standard curve or CT value and normalized to *zβ-actin* which served as the internal positive control.

mRNA In situ hybridization

Spatial localization of *foxi3a* and *foxi3b* was determined after cortisol treatment. Primer designs of digoxigenin-labeled antisense riboprobes for both *foxi3a* and *foxi3b* were similar to the previous work of Solomon *et al.,* (2003) and also subsequently used by Hsiao *et al.,* (2007). Generating anti-sense hybridization riboprobes and mRNA hybridization methods were performed as described previously (Cruz *et al.,* 2010). All images were acquired by bright-field microscope with a digital camera (Leica DFC420 C, Leica Microsystems, CH-9435 Heerbrugg, Germany).

Immunocytochemistry (ICC)

For both cortisol treatment and gene knock-down experiments, zebrafish embryos were collected and fixed with 4% parafolmadehyde in PBS solution for 2 hours. After fixation, samples were washed with PBS twice and stored in 100% methanol for future use in -20 C. Prior to ICC procedures samples were washed twice in PBST and incubated 100% ethanol for 10mins in -20 C. Several washes in PBS followed and samples were incubated to a blocking

PBST solution that contains 2% sheep serum and 2 mg/ml BSA for 4 h at room temperature. Samples are incubated at 4 C overnight in primary antibodies, anti-p63 monoclone for epidermal stem cells, anti-avian Na^+-K^+-ATPase α-subunit monoclone (NKA) for NaRC marker (α5, 1:500 dilution; Developmental Studies Hybridoma Bank, Univeristy of Iowa), anti-A-subunit of killifish H^+-ATPase polyclone (HA) for HRCs (1:200 dilution; Katoh *et al*., 2003) and including anti-phosphorylated Ser-10 of Histone 3 (PH3) polyclone to monitor cell division, diluted in blocking PBST solution. Series of washes followed, then incubation with the secondary antibodies (goat anti-rabbit/mouse IgG conjugated with Alexa Flour 568 or 488 (1:300 dilution; Molecular Probes) for 2 h in blocking solution at room temperature. Samples were washed several times with PBST and examined under confocal laser scanning microscope (TCS-SP5, Leica Lasertechnik, Heidelberg, Germany).

Morpholino Antisense Oligos Injection

GENE Tools (Philomath, OR, USA) designed and generate morpholino antisense oligos (MO) targeting the start codon (ATG) of *gr* (GR-ATG MO, 5'-CATTCTCCAGTCCTCCTTGATCCAT-3'), *mr* (MR-ATG MO, 5' ACGACATCCGATTTTGACAGTTACC-3'), and the random MO (R-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'). Followed from previous study (Matthew *et al.,* 2007) the zebrafish sequence of GR-SV MO was 5' CTGCTTCATGTATTTTAGGGTTCCG-3'. MOs were resuspended in 1X Danieau buffer: 58mmol l^{-1} NaCl, 0.7mmol l^{-1} KCl, 0.4mmol l^{-1} $MgSO_4$, 0.6mmol l^{-1} $Ca(NO_3)_2$, 5mmol l^{-1} Hepes adjusted to 7.6 pH level. Dose-dependent administration of MOs (1, 2, 4, 8ng) was administered to check the efficacy of MO. GR-ATG-MO caused severed abnormality with higher dosage, and 2 ng is the maximum dosage to allow normal development. GR-ATG MO and MR-ATG MO specificity were checked using partial sequence spanning the target site of

MO inserted into pCS2 vector with green fluorescent protein (GFP) construct. The pCS2:GFP with gene construct was sequence checked. Using SP6 mMessage mMachine kit (Ambion, Austin, TX, USA), capped mRNAs (cRNAs) were generated. cRNAs at 300pg per embryo were co-injected with or without the MO at 1 to 2 cell stages. Embryos were incubated at 29 C for further observations. Further specificity test on GR-ATG MO and MR-ATG MO were performed by western-blot analysis (Lin et al., 2011, submitted); whole-mount proteins collected from injected embryos and non-injected ones were labeled with anti-GRα and anti-MR polyclonal antibody raised in rabbit, both human origin. GR-SV MO specificity has already been established previously (Matthew *et al.,* 2007). Images were taken by Zeiss Axio Imager.M1 Upright Fluorescence microscope (Carl Zeiss Microimaging, Germany).

Cell death

Cell apoptosis was determined by terminal transferase UTP nick-end labeling (TUNEL) assayed by *in situ* cell-death detection kit (Roche Diagnostics, Indianapolis, IN, USA). Fixed embryos by 4% parafolmadehyde overnight then washed in PBS and stored in methanol. Samples were rehydrated in PBS and followed the detection kit for TUNEL assay. Positive control before the TUNEL assay was treated with DNase I for 20 min at 37 C. Samples were washed several times with PBST and examined under confocal laser scanning microscope (TCS-SP5, Leica Lasertechnik, Heidelberg, Germany).

Organ culture

Adult zebrafish were anesthetized with 0.03% MS222 (Sigma) prior to gill dissection, gills were dissected by heat sterilized dissecting materials. Samples are directly transferred to pre-incubation medium (Invitrogen, Dulbecco's Modified Eagle's Medium) containing 50mg/mL

of penicillin (GIBCO, Invitrogen), 50ug/mL of streptomycin (GIBCO, Invitrogen) and 20% Fetal Bovine Serum (FBS) (GIBCO, Invitrogen). Individual gill rakers are carefully separated from the whole gill structure. Each gill raker was cut lengthwise the filament and designated as one sample for each well (96-well). For control gill samples, freshly prepared pre-incubation medium was utilized for the entire experiment period, and for cortisol-treated group, addition of 20mg/L of hydrocortisone-21-hemisuccinate into the pre-incubation medium was utilized. Organ culture was carried out in 96-well culture plates and incubated at 28 C in humidified chamber 95% O_2 and 5% CO_2 for 1 day with medium freshly replaced twice.

Statistical analysis

Values are presented as the mean ± standard deviation (S.D.). The cortisol-treated group and the respective control group were compared using Student's *t*-test. Differences among groups were identified by one-way analysis of variance (ANOVA) (Tukey's method). A significant difference was accepted at (*) $p < 0.05$.

RESULTS

GR and MR developmental stage expression pattern

Comprehensive zebrafish *gr* gene annotation, structure characterization and expression have been previously reported (Alsop and Vijayan, 2008; Schaaf *et al.,* 2008). Gene reference for *gr* is *nuclear receptor subfamily 3, group C, member 1* (*nr3c1*), and *mr* is *nuclear receptor subfamily 3, group C, member 2* (*nr3c2*) based on NCBI and ENSEMBLE zebrafish genome database (note: *gr* and *mr* was used in this study as familiar gene reference). In the present study, more detailed gene expression during early developmental stage was demonstrated **(Figure 1)**. GR mRNA was expressed beginning at 0 hpf of embryogenesis wherein released eggs were immediately processed for total RNA collection and double-checked from templates of zebrafish eggs taken from the ovary (data not shown). The expression appears to be maternal and maintained at older stages, a similar pattern showed in the previous study of Alsop and Vijayan (2008), although their templates were taken at 1.5, 8, 25 hpf onwards, they suggested that the early *gr* expression was maternally derived also similar in mammals.

MR gene can be observed at 0 hpf closely similar of its transcript expression at 1.5 hpf previously (Alsop and Vijayan, 2008). However, in the present study *mr* expression was undetected during 2-8 hpf but resurfaced at 10 hpf onwards **(Figure 1).** This absence of *mr* expression from 2-8 hpf cannot be compared in the previous study since they collected at 1.5, 8, 25 hpf onwards, but we confirmed this absence to be precisely correct using another set of templates (data not shown).

Cortisol on zebrafish epidermal ionocyte development regulatory and marker genes

The expression of *gr* transcripts showed negative-feedback regulation after cortisol treatment demonstrating the presence of early systematic physiological regulation at 24 hpf, a natural response to maintain homeostasis **(Figure 2A).** On the other hand, *mr* did not elicit any

significant change neither stimulation nor suppression of transcript level upon cortisol treatment **(Figure 2B).**

Recent studies in zebrafish established a working model with molecular evidence demonstrating EI progenitor development, served as the basis of this study (Chang *et al.,* 2009; Ezaki *et al.,* 2009; Jänicke *et al.,* 2007; Hsiao *et al.,* 2007; Solomon *et al.,* 2003). Transcript profiles of genes involved in the EI progenitor development and genes marking particular ionocyte were screened as possible cortisol targets. NaRC marker gene *transient receptor potential cation channel, subfamily V, member 6* (*trpv6*) synonymous to *ecac* increased significantly **(Figure 3d).** Likewise, HRC marker genes such as *gcm2, solute carrier family 9 (sodium/hydrogen exchanger) member 3.2 (slc9a3.2*) synonymous to *nhe3b, and ATPase H^+-transporting lysosomal V1 subunit A (atp6v1a)* increased significantly after cortisol treatment **(Figure 3A-C).** Since *foxi3a* starts to be transcribed at the earliest time (90% epiboly) and widely expressed in different organs, qPCR analysis may not be appropriate to reflect a significant change in a specific organ. Alternatively, using whole-mount mRNA in situ hybridization, *foxi3a* detailed mRNA spatial expression can be delineated in the zebrafish embryo yolk-sac skin at 24-48 hpf parallel to 24-48 h treatment of cortisol. Significant increased in the density of *foxi3a* mRNA positive cells was observed **(Figure 4A-D, G-H).** Similarly, in the case of *foxi3b* significant increased was also observed at 48 hpf **(Figure 4E-F, I).** These data demonstrate the positive regulation of cortisol inducing regulatory genes involved in EI development particularly EI specification and differentiation evidentg of increased transcript of ionocytes marker genes.

Cortisol effect on EI development (specification, differentiation and maturation)

Several specific time and duration of treatment were set during embryogenesis to determine the precise time-frame effect and the degree of cortisol effectiveness in influencing EI

development. Unfortunately, cell density of epidermal stem cell at any given time of cortisol treatment and of duration (**Figure 5A-B)** was not affected. In contrast, both numbers of matured ionocytes NaRCs **(Figure 6A-D, I)** and HRCs increased **(Figure 6E-H, I)**. Cortisol treatment significantly increased the density of ionocytes at least 14 h treatment within the developmental stage of 1-24 hpf, an effect that could last up to 72 hpf which is the sampling period **(Figure 6-7)**. Less than 14 h treatment of cortisol could not elicit any change in ionocytes numbers for both NaRCs and HRCs (**Figure 7**).

Cell division and apoptosis after cortisol treatment

Cell division was monitored by labeling phosphorylated Histone 3 (PH3); data showed no significant increase in the rate of cell mitosis in different time-point of sampling regardless of cortisol treatment duration, 24, 48, 72 h **(Figure 8).**

Apoptosis was also tested by TUNEL assay upon cortisol treatment, 24 hpf did not show any significant difference between the WT and cortisol-treated group, however, 48 hpf showed significant decrease in cortisol-treated group **(Figure 9A-C)**. In contrast, cortisol-treated group in 72 hpf showed significant increase in apoptotic cells **(Figure 9C)**. These demonstrate exogenous cortisol significant contribution in controlling epidermal cell apoptosis.

GR and MR Loss-of-function

Cortisol regulatory effect as known to be mediated by either *gr* or *mr* was elucidated. The effect of GR-ATG MO was too strong that allows very minimal amount in achieving acceptable phenotype effect. Significant decrease in epidermal stem cell density was observed in GR-ATG MO morphants **(Figure 10B, E)**, subsequent of immense significant decreased in matured ionocytes number, NaRCs **(Figure 11C, J)** and HRCs **(Figure 12C, G)**. Using a lesser potent MO targeting the ligand-binding-domain of **GR (Matthew et al.,**

2007), GR-SV MO in similar manner dose-dependently decreased epidermal stem cells, NaRCs and HRCs density **(Figure 10C-F, 11D-J, and 12D-G, respectively).** However, loss of *mr* (MR-ATG-MO) did not show any significant change in neither epidermal stem cells **(Figure 10E)** nor HRCs and NaRCs **(Figure 11J, 12G).** However, NaRCs cell number was significantly increased at the highest dosage of MR-ATG MO (8 ng) **(Figure 11J),** a feedback that could have triggered other signaling pathway including *gr*.

The loss of function assay was further elucidated by MO injection together with exogenous cortisol treatment. Interestingly, GR-ATG MO morphants epidermal stem cell and ionocytes numbers were rescued by exogenous cortisol treatment **(Figure 13A-C,** respectively). In addition, neither of the RMO morphants, GR-ATG MO morphants with or without exogenous cortisol treatment showed increased cell division **(Figure 14)** similar to the previous data **(Figure 8).** The specificity of GR-ATG MO and MR-ATG MO were tested and found to be specific **(Figure 16).**

In vitro study on cortisol effect in gills ionocytes

To minimize other system physiological factors that would participate in the effect of exogenous cortisol, organ culture of adult zebrafish gills was performed. Density of both NaRCs and HRCs significantly increased in 1 day cortisol treatment **(Figure 17).** Both *foxi3a* and *foxi3b* transcripts in gill templates significantly increased, an indication of stimulated EI development. Whereas both *gr* and *mr* also significantly increase in transcript level an indication of functional cortisol signaling pathway in the gills **(Figure 18).** Moreover, the cortisol related enzymes called *cytochrome P450, family 11, subfamily B, polypeptide 2* (*cyp11b2*) was upregulated together with *hydroxysteroid 11-beta dehydrogenase 2* (*hsd11b2*) **(Figure 18)**. The enzyme *cyp11b2* localizes at the mitochondrial inner membrane and possess steroid 18-hydroxylase activity to synthesize aldosterone, 18-oxocortisol and steroid 11 beta-

hydroxylase activities according to NCBI gene summary database. On the other hand, *hsd11b2* encodes for the corticosteroid 11-beta-dehydrogenase activity, a microsomal enzyme complex responsible for the interconversion of cortisol to the inactive metabolite cortisone.

Cell apoptosis was also checked in the gill culture, obvious population of cell undergoing apoptosis can be observed **(Figure 19),** and however quantification is not available.

DISCUSSION

In the present study, cortisol-*gr* action is demonstrated to be significantly relevant in the EI development by stimulating *foxi3a/b* regulatory activity on epidermal ionocyte progenitor specification, differentiation and matured ionocyte proliferation. Cortisol action is clearly delineated with exogenous cortisol treatment, loss-of-function assay and in vitro study (organ culture) utilizing one of the premier model system of molecular studies, zebrafish.

In euryhaline fish, the involvement of hormonal regulation mechanism has been extensively studied like cortisol in synergy with growth hormone (McCormick and Bern, 1989; McCormick, 2001; Parks *et al.,* 2006; Perry and Gilmour, 2006). Cortisol regulation to these ion transporter genes is related to the capacity of the ionocytes for salt extrusion during seawater adaptation (Borgatti *et al.,* 1992; Kiilerich *et al.,* 2007b). In the present study, exogenous cortisol treatment significantly increased ionocytes density along with stimulated transcript levels of *ecac* the marker gene for NaRCs, *atp6v1a* and *nhe3b* for HRCs, including *ncc* for the NCC ionocytes. Similar in the case of in vitro study on FW-acclimated rainbow trout gills, transcript level of SW-associated target genes such as cystic fibrosis transmembrane conductase regulator (CFTR), Na^+,K^+,$2Cl^-$ co-transporter (NKCC), Na^+-K^+-ATPase α1b (NKAα1b), and FW-associated NKAα1a, Na^+-HCO_3^--cotransporter (NBC), and ECaC were significantly increased upon cortisol treatment (Kiilerich et a., 2011). In addition, the H^+-ATPase activity and mRNA levels in acid-base and ionic regulation is well studied to be regulated by cortisol in rainbow trout (AL-Fifi, 2006; Lin and Randall, 1993). Likewise, in the present study the H^+-ATPase sub-unit, *atp6v1a* transcript level was significantly increased upon cortisol treatment. Hence, the cortisol physiological role with that of euryhaline fishes remains faithful on regulating ionocytes function in freshwater-type fish despite of habitat preference. Whether cortisol major physiological role is for environmental

adaptation, during the early stages of zebrafish embryo development, cortisol contribution in EI development is highly regarded.

The corticosteroid signaling pathway via HPI axis begins early in the life of zebrafish and all the required components are present around the time of hatching (Alderman and Bernier, 2009; Alsop and Vijayan, 2008). Cortisol is maternally deposited as a general observation reported previously, with zebrafish *gr* mRNA transcript presence as early as 0 hpf in the present study and at 1.5 hpf previously (Alsop and Vijayan, 2008). This circumstance definitely plays a crucial role to facilitate normal embryogenesis. In the present study, *gr* down-regulated transcript was demonstrated in 24 hpf of the cortisol treated-group for 24 h (Figure 2) but not in shorter period of 14 h cortisol treatment in 14 hpf (data not shown). This manifests the presence of functional corticosteroid signaling pathway is around 24 hpf, during the completion of embryogenesis. Although stress response was observed 3-4 dpf in zebrafish previously (Alderman and Bernier, 2009; Alsop and Vijayan, 2008), in the present study, we demonstrates that exogenous cortisol was sufficient to trigger a genetic control on *gr* not related to stress. In tilapia *Oreochromis mossambicus* (Takahashi *et al.,* 2006), cortisol implantation for 7 d increased intestinal *gr* mRNA, in contrast to cortisol intraperitoneal injection decreasing *gr* mRNA, suggesting a chronic and acute mode of autoregulation, respectively. Hence, we suggest that a similar acute autoregulation is present in the case of zebrafish upon exogenous cortisol treatment around 24 hpf of 24 h cortisol treatment. On the other hand, teratogenic effects of GC has been reported (Hillegass *et al.,* 2008), abnormal craniofacial phenotypes in developing zebrafish with altered expression and activity of matrix metaloproteinases, a group of zinc-dependent endopeptidases primarily required for precise regulation of extracellular matrix degradation. The hydrocortisone dosage used was too high (275.88μM=100mg/L) compared to the present study (20mg/L). Nonetheless, we assume that the physiological effect of exogenous cortisol in zebrafish early

life development has been critically controlled by lowering *gr* transcription to ensure normal development.

EI development has been established to be facilitated by *foxi3a/3b* regulatory loop and since *foxi3a* begins to be transcribed as early as 90% epiboly hence, a good marker for tracing epidermal ionocyte progenitor (Hsiao *et al.,* 2007; Janicke *et al*., 2007). In Hsiao *et al*., (2007) demarcation of three major EI development events were established: epidermal ionocyte progenitor stage/specification (from 90% epiboly to 14-somite stage, 14s), differentiation stage (14s to 36 hpf), and maturation stage (36 hpf onwards). The early expression of *foxi3a* and *foxi3b* are timely essential as targets of exogenous cortisol action and as clearly demonstrated, transcript levels were stimulated by cortisol. In addition, the transcript of *gcm2*, an established marker for HRCs differentiation starts to express at 10 hpf (Chang *et al.,* 2009) was also increased upon cortisol treatment. On the other hand, the significant effect of exogenous cortisol in increasing ionocyte number at 72 hpf was only observed at least 14 h cortisol treatment during 1-14 hpf. It is possible that the least amount of time sufficient to potentiate EI development is 14 h, a similar event observed decades ago. For instance, in rainbow trout branchial epithelial cell (Perry and Laurent, 1989), morphological changes during the so-called "differentiation" of chloride cell occurred at least 12 h after acclimation in ion-poor-water (IPW) accompanied by transient increase in cortisol level. Similarly, significant increase in number of rainbow trout chloride cells was observed at 24 h upon transfer to IPW with cortisol treatment, however, the contrast in decreased rate of cell division (BrdUrd) starting at 12 h and became significant at 24 h was suggested to be an increase of *in situ* differentiation in epithelial stem cells (Laurent *et al.,* 1994). Moreover, in the present study, using the marker of epidermal stem cell, p63, cortisol neither stimulated stem cell proliferation nor affected rate of cell division (PH3). Hence, we propose that cortisol can boost EI development within a span of time that is sufficient to significantly

induce genes related to EI development such as *foxi3a/b* that regulates EI progenitor cells specification and differentiation.

The epidermis is a self-renewing tissue (Watt and Hogan, 2000); the EI stem cells have the capacity to proliferate and undergo terminal differentiation replenishing itself continuously (Fuchs and Raghavan, 2002), a process that cortisol-GR axis is known to be involved. For instance, in $GR^{-/-}$ knockout mice (Bayo *et al*., 2008), abnormal keratinocyte differentiation was a result of impaired activation of an apoptotic gene caspase-14, a requirement in the final step of keratinocyte differentiation. In the present study apoptosis was decreased at 48 hpf upon cortisol treatment, this is highly one possible reason of the significant increased number of ionocytes either at 48 hpf sampling time (data not shown) or at 72 hpf. In contrast, at 72 hpf, apoptosis was significantly increased to which ionocytes number remains to be significantly higher than WT. All these illustrate that cell apoptosis was delayed by exogenous cortisol, hence, boosting the increase in epidermal ionocytes density as the overall outcome in addition to its transactivational activity on *foxi3a/b.*

Unique from most teleost possessing two *gr* genes; zebrafish genome only contains one *gr* that produces two spliced variants (Schaaf *et al.,* 2008; Alsop and Vijayan, 2008). This narrows down the search on which particular receptor does cortisol mediates its effect. Zebrafish *gr* produces two isoforms from alternative splicing events producing *grα* and *grβ* (Shaaf *et al.,* 2008). To date, only human and zebrafish possess these two isoforms and very scarce data addressed this issue. *grβ* act as a dominant-negative inhibitor of *grα*, however, *grβ* do not have transactivational activity since it lacks transactivation domain (AF-2) (Schaaf *et al.,* 2008). In this study, we overcome this splice variants issue by direct assay using antisense GR-ATG-MO. Complete translation knock-down resulted to highly significant decrease number of matured ionocytes (NaRCs and HRCs) in *gr* morphants.

Evidence of *mr* role in chloride cell proliferation in freshwater rainbow trout was observed (Sloman *et al.,* 2001), likewise in the case of euryhaline killifish (Scott *et al.,* 2005). MR role was distinguished from that of *gr*, however, *mr* knock-down did not show any influence in EI development, although using highest dosage of MR-ATG MO significantly stimulated NaRCs numbers, possibly an artifact triggering other complex physiological response or absence of *mr* could have even stimulated *gr* to mediate cortisol action without the possible competition of ligand binding. Recently, in rainbow trout salinity acclimation experiments for both hyper- and hyposaline showed MR mRNA levels differential tissue-, salinity- and time-dependent changes without any changes in the case of DOC levels or DOC effect on gill ionic-related transporters (Kiilerich *et al*., 2011). It was suggested that the DOC plasma level in rainbow trout in the previous study was too low to be considered as the activating ligand for MR, hence cortisol was suggested to be the major ligand for the osmoregulatory role of MR (Kiilerich *et al*., 2011). However, Kiilerich *et al*., (2011) also mentioned about the differential tissue distribution of the 11βHSD2 may facilitate the activation of MR by DOC a normal phenomenon observed in mammals (Farman and Rafestin-Oblin, 2001; Rashid and Lewis, 2005). Although MR and GR are closely related being both activated by cortisol, their major ligands (mineralocorticoids and glucocorticoids, respectively) physiological roles are considerably different. Mineralocorticoids function is for salt, osmotic regulation and acid-base homeostasis (Stephenson *et al.,* 1984; Galaverna *et al.*, 1992) while glucocorticoids regulates catabolic responses to stress, facilitating the stress-induced peripheral carbohydrate and triglyceride metabolism, cardiovascular activity, immunity, and in brain its primary role is in neuronal regeneration, death, behaviors involved in learning, memory and adaptation (Bohn *et al.,* 1994). Apparently, epidermal ionocyte development in zebrafish would still normally proceed without *mr*. Therefore, we can assume that in the case of zebrafish skin, locally present MR or HSD11b2 is too low to interfere with

EI development and we suggest that *gr* is solely responsible if not primary receptor in mediating cortisol's mechanism of action in skin.

EI enveloping the skin embryo are the ones responsible for the ion/osmo regulation maintaining fluid homeostasis (Hwang *et al*., 1999). Similar ionocytes present in the embryo skin would eventually emerge from the functional gills becoming the main osmo/iono regulatory system of the fish. Based on this functional similarity of skin and gills, it is logical that EI development process in zebrafish embryo is similar in the case of epithelial cells in the adult zebrafish gills. In vitro, a controlled system without the influence of the systematic HPI axis, exogenous cortisol successfully stimulated increased number of ionocytes in gills filament. Similar in vitro study on chloride cell differentiation has long been performed in freshwater tilapia (McCormick, 1990). Chloride cell density was maintained at the initial number in the beginning of the experiment without increase after 4 d culture of opercular membrane (McCormick, 1990). The limitation of the in vitro technique at that time was suggested to be of missing endocrine factors. While we report only 24 h result of cortisol treatment in gills organ culture, this duration of time is sufficient to observe significant increase in ionocytes (NaRCs and HRCs) number with significant increase in *foxi3a/3b* transcripts. Notably, the gills survive the culture system till 4-6 d using anti-p63 to check the epidermal stem cells (data not shown). These results confirmed the cortisol effect in zebrafish embryo EI progenitor development. Consequently, *gr* and *mr* transcripts positive feedback regulation was observed. This increase in receptors transcripts is in accordance to accommodating more ligand supply. Notwithstanding, the *mr* increased transcripts in the gill culture assay supports the possibility that cortisol still utilizes this receptor as observed previously. Nonetheless, cortisol-*gr* axis is present locally in the gills with similar mechanism of action in zebrafish embryo.

Future work and perspectives

1. Using antagonist to further illustrate their role in the skin development; GR (RU486), MR (spironolactone and eplerenone).

2. Gain of function assay by GR and MR over-expression.

3. To identify other factors that could influence skin development such as collagen (*col1a*) and *grainyhead-like 1*(*grhl1*). Based on our micro-array analysis of templates from cortisol treated zebrafish embryo, it showed several genes up-regulated within 10, 14, and 24 h treatment, one of these genes is *col1a*. In ZFIN database, *col1a* showed mRNA localization is present in gills of zebrafish embryo. On the other hand, *grhl1* is a transcription factor highly involved in developmental processes. It was previously reported to be linked in the development of another type of non-keratinocyte epidermal cell lineage different from the *foxi3a*-dependent (Hsiao *et al.,* 2007; Jänicke *et al.,* 2007) ones in zebrafish skin. Our qPCR analysis showed both mRNA of *col1a* and *grhl1* were up-regulated after cortisol treatment for about 1-3 days parallel with stages 1-3 dpf embryo.

4. Other ways to more specifically assay cell proliferation will be explored to demonstrate more accurate results in terms of cell division or cell apoptosis. Such as using acridine orange and antibodies for caspases enzymes in the case of cell apoptosis. Cell division could be further checked by labelling cells with bromodeoxy-Uridine (BrdU).

Chapter II

Corticotropin releasing factor (CRF), CRF-receptors and related proteins contribution on skin development of zebrafish (*Danio rerio*)

Abstract

The systematic hypothalamus-pituitary-interrenal (HPI) axis was also demonstrated to play a critical role in the skin development. Loss of function assay by morpholino oligos showed *crf* and *crfr1* significantly take part in the stem cell and ionocytes density. CRFR1 is the major receptor that could mediate CRF function. Both present maternally, these may suggest their importance during the early skin development in zebrafish. CRFR2 is also maternally deposited but it can be dispensable during skin development since there is no observed change in epidermal stem cells or ionocytes density and morphology upon *crfr2* knock-down. Nevertheless, CRFR2 function cannot be ruled-out in other major physiological functions like in stress.

Altogether, the present results serve as a foundation of the HPI axis as a systematic event or locally present in the zebrafish skin and gills. In which *crf* initializes this event through *crfr1,* as a separate and local function or as a complete course of signaling events together with that of cortisol-*gr-foxi3a/3b* axis major effect in the epidermal ionocyte progenitor differentiation. More future work has to be done to clarify more issues and provide better understanding in the neuroendocrinology of the skin.

Introduction

The conventional knowledge on classical hormones having a specific function have changed parallel to advancement of new technologies and broadened understanding on molecular mechanisms behind these endocrine factors. Corticotropin releasing factor (CRF) is not exceptional to this big leap of enlightenment since its initial discovery more than 55 years ago in mammalian hypothalamus (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). CRF plays a major contribution in stress physiological response and diverse processes in vertebrates from the central nervous system (CNS) to the peripheral tissues (Turnbull and Rivier, 1997). This hormone with 41-amino acid residue possesses highly conserved signaling molecules (Chen and Fernald, 2008). In teleost fish, CRF similar to mammalian vertebrate is highly expressed in the CNS and many tissues studied, the first is in the hypothalamus region called nucleus preopticus (NPO) as the source of CRF in goldfish (Fryer and Peter, 1977), this location was confirmed by immunohistochemical and in situ hybridization in green molly (*Poecilia latipinna)* (Batten *et al.,* 1990) and *tilapia mossambicus* (Pepels and Balm 2004) including *crf* mRNA expression in rainbow trout (Ando *et al.,* 1999). Aside from *crf* major expression in the CNS including spinal cord, it is also expressed in retina, muscle, gills, spleen, intestine, liver, kidney, ovary and heart in African cichlid *Astatotilapia burtoni (A. burtoni)* (Chen and Fernald, 2008). In common carp (*Cyprinus carpio* L.), *crf* thorough expression in the brain was shown, also its presence in gills, head kidney, kidney, testis, structural characterization were elucidated (Huising *et al.,* 2004; Huising *et al.*, 2007) including in skin (Mazon *et al.,* 2006). Likewise, zebrafish *crf* distribution in the brain by whole mount in situ hybridization and histochemistry were shown together its expressions in tissues like eyes and gills, noting that very few tissues were tested (Chandrasekar *et al.,* 2007). Noticeably, the high expression of CRF in fish gills is very apparent to all species studied, signifying a primary role to the major physiological activity of

fish survival, osmo-/ionoregulation. Gill cells called ionocytes are the ones carrying-out this function (Hwang *et al.,* 2011) and these similar cells can be found in the skin of fish. Moreover, endocrine control plays a major part in these adaptational skills of fish (McCormick, 2001).

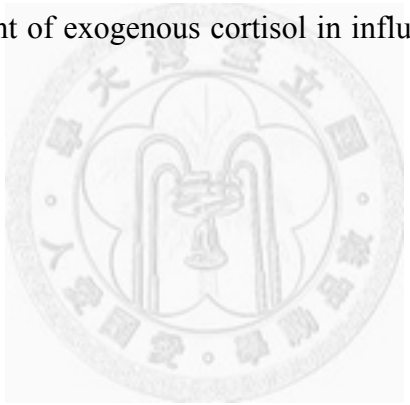
In mammalian skin, CRF is present together with its receptors and related proteins (Karalis *et al.,* 1991; Slominski *et al.,* 1995, 1998, 1999, 2001). By the use of reverse-phase high-performance liquid chromatography (HPLC) and CRF radio immunocytochemistry (RIA), actual CRF in rodents and mouse is at 36 and 67 fmol/g of wet tissue in telogen and mid-anagen stages of the skin (Roloff *et al.,* 1998). Mouse and rat CRF in the skin can be located in pilosebaceous unit of the hair follicle, to keratinocytes of the outer root sheath (ORS), matrix region of the developing hair follicles, basal epidermis, dermal region and sub-cutaneous compartments (Roloff *et al.,* 1998, Slominski 1996, Karalis *et al.,* 1991). In human, mRNA of CRF can be tested in normal and pathological skin specimens, cultured normal and malignant keratinocytes and melanocytes, other melanoma cell lines, cultured junctional and dermal nevocytes, epidermal melanocytes, epidermis, hair follicles, and squamous carcinoma cells together with actual production of CRF peptide in these tissues (Slominski *et al.,* 1995, 1996, 1998). Furthermore, human CRF in skin was detected by immunocytochemistry and in situ hybridization in melanoma specimens, pilosebaceous units and nevocytes (Funasaka *et al.,* 1999; Kono *et al*., 2000). The supply of CRF in the skin is not only from cutaneous production but also from skin neural ends (Slominski and Wortsman, 2000). Moreover, the skin neuroendocrine system was suggested to function by preserving and maintaining cutaneous structural and functional integrity and systemic homeostasis in inducing vascular system, immune system, and/or pigmentation changes when exposed to foreign or local stimulation (Slominski and Wortsman, 2000). Hence, it is worthy

to elucidate the role of CRF in skin development considering its major physiological importance in the survival of fish.

The regulation of the hypothalamus-pituitary-adrenal (HPA) in human or HP interregnal (HPI) in fish axis is well understood and research development in this field is continuously progressing. Normally, by environmental stimulation such as stress, this axis will be stimulated and regulated (Chrousos and Gold, 1992); stimulates the production and release of CRF from central circuits in the hypothalamus mediated by its receptors (CRFR1 and CRFR2) which are present in the pituitary (Perrin and Vale, 1999). This signal transduction pathway could result to the production and release of adrenocorticotropic hormone (ACTH) (including β-endorphin) from the anterior pituitary (Smith and Founder, 1988). Endocrine ACTH derived from pre-opiomelanocortin peptide (POMC) mediates its effect on *melanocortin 2 receptor* (MC2R) that belongs to the family of G protein-coupled receptors (Perrin and Vale, 1999; Logan *et al.,* 2003) present in adrenal gland. The adrenal gland in human and called interrenal tissue in fish would respond to this stimulation, produces and secretes glucocorticoid hormone (cortisol in human and fish, cortisone in rats) (Mommsen *et al.,* 1999) that would ultimately act on the initial stimulation which is stress as an example mentioned above. CRF high potency in corticotropin (ACTH) releasing activity is notable since then, based on assay using cultured rat pituitary cells upon characterization of its primary structure in ovine (sheep) hypothalamus (Spiess *et al.,* 1981). Interestingly, all these components, related proteins and signaling transduction were all present in mammals (Ito *et al.,* 2009; Slominski and Wortsman, 2000; Slominski *et al.,* 2001, 2006, 2007) and fish skin or in gills (Alaru *et al.,* 2008; Chen and Fernald, 2008; Mazon *et al.,* 2006; Metz *et al.*, 2005, 2006).

In zebrafish, *crf* participates and plays a critical role in the camouflage response on melanocytes, with sensitiviy in ethanol treatment and light exposure (Wagle *et al*., 2011).

This is the only study showing CRF role in the skin of teleost fish, and no other available study to demonstrate its function in other aspect of skin. Using zebrafish as the model system, the present study is the first attempt to elucidate the molecular mechanism on local signaling pathway of CRF and related proteins in skin development. In connection to the chapter I of the present study, *crf* and related proteins together with exogenous cortisol aim to illustrate the complete HPI axis role; this will serve as a foundation of its novel physiological function in zebrafish skin development. This chapter extends the scope of the part I study in clarifying specific issues such as (1) *crf* role in skin development particularly ionocyte progenitor specification, differentiation and proliferation, (2) identifying the major *crf* receptor mediating its action as it possess 2 receptors known for their distinct binding capacity with other ligands, and (3) the extent of exogenous cortisol in influencing *crf* expression together with related proteins.



Materials and Methods

(NOTE: Most of the experimental procedures/assays and statistical analysis for this chapter were similar to the previous chapter. Please refer to the previous Materials and Methods described above)

Reverse-Transcription PCR (RT-PCR)

Total RNA in several developmental stages was isolated from zebrafish embryos: 0 (immediately processed when released from the female zebrafish), 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 36 hours post fertilization (hpf). Similar to tissue scan samples, the following tissues were collected: brain, eyes, gills, heart, intestine, liver, spleen, intestine, kidney, skin and muscle. Transcript expressions of the following genes are determined: *crf, crfr1, crfr2, mc2r* and *β-actin* served as the internal positive control. All gene sequences were gathered from NCBI and Ensemble genome databases. All primers used for the whole study were designed using Primer Express software (version 2.0.0; Applied Biosystems). Primers used are listed at TABLE 1. The primer products were amplified by a conventional reverse-transcription polymerase chain reaction (RT–PCR). Analysis was performed with 5 min of denaturation at 95 C, followed by 30 s at 95 C. The annealing time was 30 s at 55~59 C based on the melting temperature of the primers used, and the elongation period was 30~60 s depending on the length of the product at 72 C. All reactions were run for 30 cycles. Amplified transcripts were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA), and all amplicons were sequenced to confirm the desired products.

Quantitative real-time PCR (qPCR)

After cortisol treatment, mRNA expressions of target genes were measured by qPCR using ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA) and Universal SYBR Green master mix (Applied Biosystems). Primers of *crf*, *crfr1*, *crfr2*, *mc2r*

and crf binding protein (crfbp) were listed in TABLE 2. All qPCR reactions were performed as follows: 1 cycle of 50 C for 2 min and 95 C for 10 min, followed by 40 cycles of 95 C for 15 s and 60 C for 1 min. Control reactions used sterile water to determine background and genomic DNA contamination levels. Values were computed using a standard curve or CT value and normalized to *zβ-actin* which served as the internal positive control.

Morpholino Antisense Oligos Injection

GENE Tools (Philomath, OR, USA) designed and generate morpholino antisense oligos (MO) targeting the start codon (ATG) of the random MO (R-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'), *crf* (CRF-ATG MO, 5'-TGAGCTTCATGTCAAAAAGTGGCGA-3'), *crfr1* (CRFR1-ATG MO, AGCATCTTTTCATCCTTTCATCCAT), *crfr2* (CRFR2-ATG MO, GCTCGACGTTATTCCAGAATTCCAT), and *mc2r* (MC2R-ATG MO, CATGATGGATCGATGATCACTCTTA). MOs were resuspended in 1X Danieau buffer: 58mmol l^{-1} NaCl, 0.7mmol l^{-1} KCl, 0.4mmol l^{-1} $MgSO_4$, 0.6mmol l^{-1} $Ca(NO_3)_2$, 5mmol l^{-1} Hepes adjusted to 7.6 pH level. Dose-dependent administration of MOs (1, 2, 4, 6, 8ng) was administered to check the efficacy of MO. CRFR1-ATG-MO caused severed abnormality with higher dosage, and 2 ng is the maximum dosage to allow normal development. CRF-ATG-MO and CRFR2-ATG-MO was too weak to cause any abnormality evan the use of 8 ng MO. Specificity check using partial sequence spanning the target site of MO inserting into pCS2 vector with green fluorescent protein (GFP) construct is still on its way. The pCS2:GFP with gene construct was sequence checked. Embryos were incubated at 29 C for further observations.

Results

CRF, CRF receptors and ACTH receptors gene profiles

Ensembl genome database for zebrafish showed one CRF gene located at chromosome 24, gene accession no. ENSDARG00000027657 with 3 transcripts (1079 bp, 162 aa, ENSDART00000038290; 420 bp, 100 aa, ENSDART00000145712; 931 bp, 162 aa, ENSDART00000133649), multiple alignment could show that they possessed 100% if not high sequence identity, and so *crf* ENSDART00000038290 having the longest length of transcript was utilized for this study. NCBI genome database predicted zebrafish *crhr1* gene located in chromosome 3 with 1620 bp, 539 aa residue and a product name of *crfr1* (hereafter, *crfr1* will be used as reference to *crhr1* gene). For the second CRF receptor gene, predicted *crfr2* is located at chromosome 2 with 1173 bp transcripts and 390 aa translated residue.

Gene expression of *crf*, *crfr1* and *crfr2* can be detected as early as 0 hpf onwards **(Figure 1)** a similar pattern reported previously **(**Alderman and Bernier, 2009**);** maternal expression was suggested. Tissue distribution for *crf* mRNA can be found almost to all tissues tested such as brain, eyes, gills, heart, spleen, intestine, skin, muscle except in liver and kidney **(Figure 2)**. In the case of *crfr1*, mRNA expression is in brain, eyes, gills, heart, liver, skin and muscle except in spleen, intestine, and kidney **(Figure 2)**. Moreover, *crfr2* can be found in brain, eyes, gills, heart, spleen, kidney and skin except in liver, intestine and muscle **(Figure 2)**.

ACTH receptor (*acthr*) is known as *melanocortin 2 receptor gene* (*mc2r*), from hereafter, *mc2r* will be used as reference instead of *acthr.* Ensembl genome database showed zebrafish *mc2r* is located at chromosome 16 with 1236 bp transcripts and 304 aa translated residue. In the case of *mc2r*, showed multiple bands out of several primer designs (data not shown), correct band size was cloned and sequenced, and was shown to be correct *mc2r*

transcript. This implies that the predicted *mc2r* gene in zebrafish genome is truly present, as *mc2r* structure and evolution in zebrafish has been previously characterized (Logan et al., 2003) including knock-down and transcript expression during interrenal development (Thahn *et al.*, 2007).

Exogenous cortisol treatment of zebrafish embryo

The exogenous cortisol treated-group at 24 hpf showed significant elevated *crf* mRNA, however, at 72 hpf no comparable difference to WT was observed **(Figure 3A).** In the case of the *crfr1*, no significant change was observed but only apparent decrease at 72 hpf **(Figure 3B).** However, *crfr2* mRNA was significantly down-regulated in the cortisol-treated group compared to WT at 72 hpf **(Figure 3C),** demonstrating a clear negative-feedback when cortisol level exceeds the normal level. Moreover, mc2r showed significant decreased both at 24 and 72 hpf **(Figure 4)**, similar to *crfbp* significant decrease at 24 hpf **(Figure 5).**

Loss-of-function Assay

The knockdown of *crf* translation by CRF-ATG MO did not elicit severe change with undetectable signs of any morphological disturbance. However, the epidermal stem cell was significantly decreased in cortisol-treated group compared to RMO which is parallel to increased MO dosage starting from 2 ng and aggravated at 4-8 ng CRF-ATG MO **(Figure 6).** This issue was corrected by exogenous cortisol treatment among CRF-ATG MO morphants. Epidermal stem cells recovered to normal number by exogenous cortisol treatment **(Figure 7A).** In the case of ionocytes upon *crf* gene knockdown, neither increase nor decrease was observed in NaRC **(Figure 8A)** and HRC **(Figure 8B)** density regardless of CRF-ATG MO dosage. However, despite of exogenous cortisol among *crf* morphants, HRC density was not

elevated unlike in the case of RMO morphants with significant increased numbers **(Figure 7B)**. This could imply the severity of loss epidermal stem cells among morphants.

In CRFR1-ATG MO morphants, both epidermal stem cells and ionocytes were affected **(Figure 9-10)**. Dosage of CRFR1-ATG MO cannot be raised higher than 2 ng due to its toxicity level in embryos however; this amount is acceptable enough not to cause morphological abnormality. Results showed that the epidermal stem cell was significantly decreased among CRFR1-ATG MO morphants **(Figure 9A, 10)**. Similarly, both ionocytes NaRC and HRC were significantly decreased **(Figure 9B, C, 10)**. In contrast, epidermal stem cells and ionocytes of CRFR2-ATG MO morphants was not affected **(Figure 11)**. Double injection of both receptors MOs did not elicited additive decreased shown initially among CRFR1-ATG MO morphants **(Figure 10)**. These implies that *crfr1* could be the major receptor in mediating *crf* effect in controlling epidermal cells differentiation or proliferation.

The MC2R-ATG MO did not elicit any decrease or increase in epidermal stem cells and ionocytes among its morphants **(Figure 12).**

Gene expressions after gene knock-down with exogenous cortisol treatment

Loss of *crf* stimulated both *crfr1* and *crfr2* mRNA level at 4ng of CRF-ATG MO, however, this stimulation returned to normal expression level with higher MO dosage at 6ng **(Figure 13).** Similar trend was observed for both *mr* and *gr* mRNA expressions in CRF-ATG MO morphants, stimulated at 4ng MO and returned to normal level at 6ng CRF-ATG MO, while *cyp11b2* was continuously upregulated with the increasing dosages of CRF-ATG MO. However, *foxi3a*, *foxi3b* and *hsd11b2* were not affected by the loss of *crf*.

In CRFR1-ATG MO morphants, mRNA expression level of *crfr1*, *crfr2*, *crf*, *gr* and *mr* were not affected except for both *foxi3a* and *foxi3b* suppressed expression **(Figure 14).** These results imply that epidermal development particularly the differentiation of ionocyte

progenitor was severely compromised as observed among CRFR1 morphants with decreased ionocytes number.

Cell division

Cell mitosis was not affected among morphants of CRF-ATG **(Figure 15),** CRFR2-ATG **(Figure 11)** and MC2R-ATG **(Figure 12)** MOs, except for CRFR1-ATG MO with significant increase **(Figure 15)** and although not significant this increase was already obvious in previous data **(Figure 10)**. However, this increase on cell division among CRFR1-ATG MO morphants cannot be further increased upon cortisol treatment.

Discussion

The role of CRF as the initial signaling factor in the HPI axis was demonstrated for the first time to influence several issues in zebrafish skin development. In the present study, *crf* takes part on epidermal stem cell density, and *crfr1*is suggestive to play the major role in mediating CRF function. Since the loss of *crfr1* alone could elicit immense disturbance in the epidermal cell density including stem cells and ionocytes. These results suggest that not only *crf* and *crfr1* contribute to zebrafish skin development, but also some related peptide that could recognize CRFR1 might have contributed to this process.

The tissue distribution of zebrafish *crf* is most likely similar to other teleost fish and vertebrates and almost ubiquitously distributed. For instance, in this study zebrafish *crf* was absent in liver and muscle but present in one of the developed model system, African cichlid *A. burtoni* except in its pituitary, testis and stomach (Fernald, 2003; Chen and Fernald, 2008) (pituitary and testis are not included in present study while stomach is absent in zebrafish). Notably, previous study in zebrafish demonstrated that *crf* is only expressed in the brain, eyes, and gills (Chandrasekar *et al.,* 2007). In the present study, additional *crf* expression was shown in heart, spleen, intestine, skin, and muscle, apparently in minimal level. Hence, this could have been due to the difference of *crf* primers used or other factors. Nevertheless, this high tissue distribution rate of *crf* mRNA may prove to illustrate its high physiological significance. On the other hand, zebrafish *crfr1* is not present in intestine and kidney but both present in *A. burtoni* with absence in spinal cord, stomach, liver, ovary, testis and heart (Chen and Fernald, 2008), in contrast to zebrafish *crfr1* presence of expression in liver and heart in the present study. In addition, zebrafish *crfr2* absence in liver and muscle is in contrast to its presence in *A. burtoni.* These differences may reflect evolutionary significance based on environmental adaptation and species specificity. Nevertheless, the tissue

distribution of both ligand *crf* and receptors (*crfr1* and *crfr2*) demonstrate highly functional proteins with considerable physiological significance.

The potency of CRF-ATG MO is obviously not strong enough to elicit such great effect on skin development. This could be due to related peptide that is well-known to have similar physiological effect like CRF, the urotensin I (Arnold-Reed and Balment, 1994; Bernier 2006, Koob and Heinrichs 1999; Lederis *et al*., 1985), it evokes the release of ACTH and could highly bind to CRF receptors specifically *crfr2*, notably 10 times more potent compared to CRF (Vaughan *et al.,* 1995). Urotensin I is present in zebrafish embryo approximately from 6 hpf later than *crf*, *crfr1* and *crfr2* which are all present from 0 hpf (Alderman and Bernier, 2009). Hence, knock-down of *crf* could have hardly made a difference since urotensin I could compensate for the compromised work of CRF.

Morphants of CRFR1-ATG MO showed significant loss of both the epidermal stem cells and ionocytes. It illustrates that *crf* signal is highly mediated by *crfr1*, and could not be replaced by the existing alternative way using *crfr2*, since *crfr2* loss of function showed no apparent change in epidermal cell development. These suggest *crf* function mediated by *crfr1* is the major player in the zebrafish skin development. In addition, double knock-down of both receptors did not aggravate the decrease of ionocytes demonstrated in CRFR1-ATG MO morphants. This may suggest that other factors compensate for the loss, as mentioned above.

The CRF binding protein (CRFBP) was known to participate and regulate CRF physiological effects (Potter *et al.,* 1992; Westphal and Seasholtz, 2006; Boorse *et al.,* 2006).CRFBP mRNA is also present in 0 hpf in zebrafish newly fertilized embryo and expressed highly in the brain at all stages (2-5 dpf) (Alderman and Bernier, 2009). Amount of free CRF and signaling is regulated by CRFBP by sequestering CRF, decreasing the level of free CRF from the circulation and preventing receptor binding (Boorse *et al.,* 2006; Petraglia *et al.,* 1993; 1996). Hence, CRF loss of function may have driven CRFBP to free

existing bound CRF to rescue the abnormality. Not to mention, maternal cortisol is readily available to impart its shares in the proper skin development, supposedly initially signaled by *crf*.

In relation to the chapter I, CRF as the initiator of cascading signals in the HPI axis is proven in the present study to highly respond in exogenous cortisol effect. Exogenous cortisol treatment for 24 h parallel to 24 hpf developmental stage as the sampling period showed significant increase in *crf* mRNA while at the same time *crfbp* showed significant decrease. This shows that exceeding high amount of cortisol should be regulated and is best served by *crf* to ensure homeostasis. Although *crfr1* and *crfr2* showed apparent and significant decrease at 72 hpf, this latency of down-regulation is a signal of negative-feedback for the earlier increase of *crf* mRNA. In addition, *mc2r* mRNA, one of the down-stream pathways of *crf* signal, significantly decreases promptly at 24 h cortisol-treated group (24 hpf development stage) up to the 72 h sampling time (72 hpf developmental stage). More so, the loss of *gr* showed increased *crf* mRNA expression along with *crfbp* in templates of *gr* morphants. These results demonstrate the complete closed-loop of signals, a completion of the HPI axis.

The role of CRF in skin development is highly important in stem cell only, but with the loss of *crfr1* it became clearer that *crf* effect extends to the ionocyte progenitor development as well. Notably, for both *crf* and *crfr1* morphants the decrease in epidermal stem cells and ionocyte decrease, respectively, cannot be rescued by exogenous cortisol treatment. For these, we can only suggest that the loss of these genes is highly important at the epidermal stem cells to begin with and this would eventually dictate the faith of the ionocyte progenitor density. In addition, among the *crfr1* morphants, the cell division increases significantly, probably a compensatory effect for the decreased epidermal stem cell density. But this course of action is not sufficient enough to boost the density of developing ionocyte progenitor, hence, the ionocyte number among *crfr1* morphants still showed

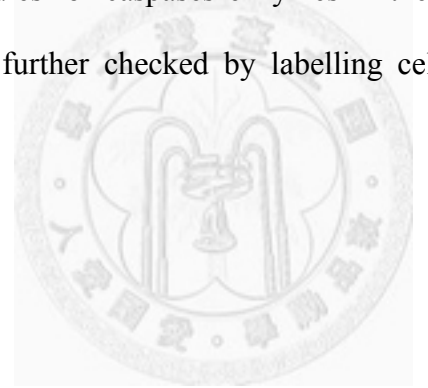
significant decrease. Nevertheless, *crf – crfr1* course of action in skin development could be considered as a separate entity from that of complete HPI axis role. Altogether, local and systemic effect of *crf* down to cortisol is demonstrated to be essential in zebrafish skin epidermal development.

Future work and perspectives

1. Using antagonist to further illustrate their role in the skin development; astressin is a non-selective CRFR1/R2 antagonist, and antalarmin is a potent CRFR1 antagonist and K41498 is for CRFR2 antagonist
2. Gain of function assay by CRF, CRFR1 and CRFR2 over-expression.

3. To explore the deeper role of other related proteins like CRFBP and Urotensin I in skin development.

4. Other ways to assay cell proliferation will be explored to demonstrate more accurate results in terms of cell division or cell apoptosis. Such as using acridine orange and antibodies for caspases enzymes in the case of cell apoptosis. Cell division could be further checked by labelling cells with bromodeoxy-Uridine (BrdU).



PART I-Conclusions

The existing and improving epidermal developmental models in teleost fish especially in zebrafish helped tremendously to realize this study. Critical points starting from epidermal stem cells, epidermal progenitor development (specification, differentiation and maturation), and terminal differentiation that leads to programmed cell death including cell division and cascades of signals of the typical HPI axis were elucidated. First, the present study established the molecular signaling pathway of cortisol-*gr* axis, a tremendous involvement in the event of skin epidermal development with *foxi3a/3b* as target genes and delaying apoptosis boosting the end result of development, matured ionocyte increased proliferation (Figure C1-2, A). This axis could be present both in epidermal stem cell and epidermal ionocyte progenitor cells, and eventually impacted the proliferation of individual ionocytes studied. Secondly, the major role of CRF in epidermal stem cells density was demonstrated to be of primary necessity in dictating the subsequent condition ensuring proper stages of epidermal ionocyte progenitor development, mediated primarily by *crfr1*. This axis could be locally present in the skin notwithstanding the greater involvement of the whole system. Exogenous cortisol treatment could elicit a negative-feedback signal to the CNS where CRF responds to initiate homeostasis down to related proteins/enzymes and tissues involved. Altogether, these factor and related peptides down to cortisol whether as a single signal or as a complex network were proven to be of critical importance in the zebrafish early skin development (Figure A). This study contributes to the novel physiological roles of highly important endocrine factors in teleost fish and more work to be done to establish a better understanding in zebrafish skin development.

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PART II

Chapter I

Ghrelin affects carbohydrate-glycogen metabolism via insulin inhibition and glucagon stimulation in the zebrafish (*Danio rerio*) brain

Abstract

Carbohydrate-glycogen metabolism (CGM) is critical for emergency energy supplies in the central nervous system (CNS). Ghrelin (GHRL) in pancreas is known to significantly regulate a dominant player in CGM, insulin (INS). However, its regulatory effect on extrapancreatic INS synthesis is yet unknown. In this study, we used adult zebrafish to elucidate the expression and role of zebrafish GHRL (zGHRL) in genes primarily involved in the brain's CGM. Results showed that zebrafish brain expressed *zghrl* and its receptor, growth hormone secretagogue-receptor (GHS-R: *zghs-r1a* and *zghs-r2a*), according to RT-PCR and *in situ* hybridization. Protein localization coupled with mRNA spatial expression further verified zGHRL's presence in the brain. For the *in vivo* study, significant increases in *zghs-r1a* and *zghs-r2a* synthesis were observed after injection of synthetic peptide goldfish GHRL-12 (gGHRL) using brain templates analyzed by quantitative real time-PCR (qPCR). Ligand-receptor synthesis of INS (*zinsa; zins-r1* and *zins-r2*) significantly decreased, while glucagon (GCG) (*zgcgb1* and *zgcgb2; zgcg-r1* and *zgcg-r2*) exhibited a significant transient increase. In CGM, subsequent processes indicate urgent glucose-sensing response that will balance glycogen degradation and energy storage. Taken together, these findings suggest that GHRL

regulates INS synthesis by mediating its action on GHS-R in the CNS and partly involved in CGM.

Introduction

After the invention of synthetic peptides known as "growth hormone (GH) secretagogues" that can stimulate the release of GH (Bowers *et al*., 1977; Bowers *et al*., 1980), GH secretagogue receptors (GHS-R1a and GHS-R1b) were discovered by utilizing an expression cloning strategy (Howard *et al.*, 1996). The first and extensively characterized natural ligand for orphan receptor GHS-Rs was ghrelin (GHRL) (Kojima *et al.,* 1999). Both mammals and fish show highly conserved GHRL gene organization and similar regulatory actions. GHRL is widely distributed in almost all tissues (Gnanapavan *et al.,* 2002). GHRL was extensively studied in such fish as goldfish (Matschinky, 1996; Miura *et al.*, 2006; Unniappan and Peter, 2004), Mozambique tilapia (Riley *et al.*, 2002), Nile tilapia (Parhar *et al.,* 2003), eel (Kaiya *et al.,* 2003b, 2006), rainbow trout (Kaiya *et al.,* 2003a; Sakata et al., 2004), channel catfish (Kaiya et al., 2005) and seabream (Yeung *et al.,* 2006). In addition, zebrafish GHRL (zGHRL) gene organization was also described (Kojima and Kangawa, 2005). Previously, a feeding experiment in zebrafish showed increased brain and gut zGHRL messenger (m)RNA synthesis with 3~7 days of fasting, but it was significantly downregulated once feeding resumed (Amole and Unniapan, 2008). GHRL demonstrates a wide array of biological actions; however, a role in energy metabolism and the vast cascade of downstream mechanisms has not been clearly delineated.

The high demand for energy by the brain is efficiently met by glucose; the uptake of this major metabolic energy substrate is primarily signaled by insulin (INS). INS is one of the major energy metabolic factors that are reportedly regulated by GHRL (Cui *et al.*, 2008; Dezaki *et al.*, 2008; Vestergaard *et al.,* 2008). For instance, GHRL administration in healthy humans leads to hyperglycemia and reduced INS secretion (Broglio *et al.,* 2004), while other reports showed stimulatory INS secretion in the pancreas of normal and diabetic rats (Adeghate and Ponery, 2002). Moreover, based on results of combined *in vitro* and *in vivo*

studies of ghrelin's effects on insulin release in isolated pancreas islets and intact mice, inhibition of glucose-induced INS release by GHRL was suggested to be mediated by phospholipase C pathways, while stimulatory effects of high dosages of GHRL occur through cyclic AMP in isolated mouse islets (Salehi *et al.,* 2004).

Previous studies demonstrated the presence of GHRL in the pancreas (Chanoine and Wong, 2004) with the GHS-R (Guan *et al.,* 1997; Volante *et al.,* 2002). In zebrafish, zGHRL was used as a marker in an endocrine pancreatic developmental study after the knock-down of *nkx2.2a*, a transcription factor crucial for pancreatic organ development (Pauls *et al.,* 2007). In addition, GHRL affects the secretion of several pancreas-derived hormones as demonstrated in rat and mouse isolated islets (Irako *et al.,* 2006; Lee *et al.,* 2002, Qadar *et al.,* 2007). On the other hand, the extrapancreatic production and secretion of INS, for instance in the brain and pituitary cells (Budd *et al.,* 1986), has been debated in higher vertebrates and yet remains controversial (Gerozissis, 2003). In teleost fish, the mRNA of the *insulin-b* isoform was localized in the brain while the *insulin-a* isoform was expressed only in the pancreas during embryonic stages of zebrafish (Papasani *et al.,* 2006). In tilapia, INS transcripts in the brain and pituitary cells were also identified (Hrytsenko *et al.,* 2007).

Glucagon (GCG), the counter-regulatory hormone of INS is also associated with GHRL. In the rat stomach, GHRL and GCG receptor proteins were found to co-localize in a few cells (Katayama *et al.,* 2007). GCG was demonstrated to stimulate GHRL secretion in the isolated rat stomach (Kamegai *et al.,* 2004). A reverse pathway by which GCG can be inhibited or stimulated by GHRL was also suggested (Katayama *et al.,* 2007). For instance, GHRL's stimulatory effect on GCG secretion was demonstrated in pancreatic fragments, from diabetic rats, which had been incubated in a GHRL solution (Adeghate and Parvez, 2002). Furthermore, other metabolic factors that contribute to carbohydrate homeostasis such as glycogen synthase (GS), glycogen phosphorylase (GP), and the glucose transporter

(GLUT) are known to play key roles in CGM among vertebrates. So far, the proposed mammalian model extensively described for the glucose-sensing system is that of pancreatic tissue (Matchinsky, 1996). In this model, glucose uptake is yielded by the GLUT upon INS metabolic action followed by subsequent metabolic processes (Thorens, 2000). Likewise, regulatory and transport proteins participate in glycogen metabolism and transport of metabolites as described in the fish liver and gill cells (Tseng and Hwang, 2008). Altogether, a possible link between GHRL and these metabolism-related proteins may provide a better understanding of the maintenance of the energy supply and energy storage in the brain.

In this study, we explored the role of the zGHRL-zGHS-R system in the CNS complex regulatory network for CGM. We demonstrate that in zebrafish, exogenous gGHRL can mimic systemic zGHRL production which suppresses INS synthesis thereby leading to the urgent expression and regulation of metabolism-related genes; *zins-r, zgcg*, *zgcg-r*, *zgp*, *zgs,* and *zglut*. Therefore, we suggest that GHRL has a significant influence on energy metabolism, and we provide additional elucidation of its role and biological importance.

Materials and methods

Experimental animals

Zebrafish (*Danio rerio*), 400~500 mg in body weight, were obtained from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. The fish were reared in a circulating system containing local fresh water at 27~28 °C under a 14: 10-h light: dark photoperiod. Daily feeding consisted of artificial feed pellets (Fu-So, Taipei, Taiwan). The Academia Sinica Institutional Animal Care and Utilization Committee approved the experiment protocols used in this study.

Total RNA isolation and complementary (c)DNA synthesis

Total RNA isolation followed the standard protocol with the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Prior to isolation, fish were anesthetized with 0.03% MS222. Adult zebrafish tissue samples were homogenized in 1 ml of Trizol reagent per 50~100 mg of tissue. Total RNA was purified by a MasterPure^{TM} RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). The total amount of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from the purified and quantified total RNA using the SuperScript^{TM} kit for reverse-transcription polymerase chain reaction (RT-PCR; Invitrogen).

mRNA expression in tissues

Total RNA of the following tissues was isolated from adult zebrafish: brain, eyes, gills, heart, liver, spleen, intestines, kidneys, skin, and muscles. Tissue distributions of the following genes were determined: *zghrl*, *zghs-r1a*, *zghs-r2a*, *zins-r1*, *zins-r2*, *zgpb*, *zgpl*, *zgpm1*, *zgpm2*, *zgsm*, *zgsl* (liver), and *zβ-actin* which served as the internal positive control. Gene distributions of *zglut* isoforms (*zglut1a, zglu1b, zglut8,* and *zglut13.1*) included in this study

are from a previous study (Tseng and Hwang, 2008). Only the genes and isoforms that were expressed in the brain were chosen for further studies.

The NCBI and Ensemble gene databases were searched for target gene sequences. The primers used are listed in **Table 1** and for some target genes (*zghs-r1, zghs-r2a*, *zins-r1* and *zins-r2*) utilized the qPCR primers listed in **Table 2**. The primer products were amplified by a conventional reverse-transcription polymerase chain reaction (RT–PCR). Analysis was performed with 5 min of denaturation at 95 °C, followed by 30 s at 95 °C. The annealing time was 30 s at 55~59 °C based on the melting temperature of the primers used, and the elongation period was 30~60 s depending on the length of the product at 72 °C. All reactions were run for 30 cycles. Amplified transcripts were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA), and all amplicons were sequenced to confirm the desired products.

Cryosections

The whole brain was surgically removed from an adult zebrafish (6 months old) after being anesthetized with 0.03% MS222. The whole brain was fixed in 4% paraformaldehyde at 4 °C for 4 h. Samples were washed in phosphate-buffered saline (PBS) with Tween-20, PBST (0.2% Tween 20, 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na_2HPO_4, and 0.002 mM KH_2PO_4; pH 7.4) several times and gradually immersed in PBS containing 5%, 10%, and 20% sucrose for 10 min each step at room temperature. In the final incubation, samples were soaked in PBS containing OCT compound (Sakura, Tokyo, Japan) and 20% sucrose (1: 2) overnight at 4 °C. At the beginning of cryosectioning, samples were embedded in 100% OCT compound medium at -20 °C. Brain sections were cut as either cross- or sagittal-sections at approximately 10-μm thick using a cryostat (CM 1900; Leica, Heidelberg, Germany). Brain

sections stuck on poly-l-lysine-coated slides were used both for mRNA *in situ* hybridization and immunocytochemistry.

In situ hybridization

Localization of the target genes, *zghrl*, *zghs-r1a,* and *zghs-r2a*, was investigated. Purified plasmids obtained from the primer set listed in **Table 1** were utilized to synthesize cRNA probes. Purified plasmids were linearized by restriction enzyme digestion, and *in vitro* transcription was performed with T7 and SP6 RNA polymerase (Roche, Penzberg, Germany) in the presence of digoxigenin (dig)-UTP. Dig-labeled cRNA probes were examined using RNA gels and a dot-blot assay to confirm the quality and concentration. For the dot-blot assay, the synthesized probes and standard cRNA probes were spotted on nitrocellulose membranes according to the manufacturer's instructions. After cross-linking and blocking, the membrane was incubated with an alkaline phosphatase-conjugated anti-DIG antibody and stained with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Prepared cryosection slides of adult zebrafish brain were rinsed with PBST, and incubated with hybridization buffer (HyB) containing 60% formamide, 5× SSC, and 0.1% Tween 20 for 5 min at 65 °C. Pre-hybridization was performed with HyB^+, which is HyB supplemented with 500 μg/ml yeast transfer (t)RNA and 25 μg/ml heparin, and then slides were incubated for 2 h at 65 °C. After pre-hybridization, 100 ng of the cRNA probe in 300 μl of HyB^+ were used for hybridization at 65 °C overnight. Samples were then washed at 65 °C for 10 min in 75% HyB and 25% 2× SSC, 10 min in 50% HyB and 50% 2× SSC, 10 min in 25% HyB and 75% 2× SSC, 10 min in 2× SSC, and two times for 30 min in 0.2× SSC at 70 °C. Further washes were performed at room temperature for 5 min in 75% 0.2× SSC and 25% PBST, 5 min in 50% 0.2× SSC and 50% PBST, 5 min in 25% 0.2× SSC and 75% PBST, and 5 min in PBST. Samples were next incubated in blocking solution containing 2% sheep serum and 2

mg/ml BSA in PBST for 2 h and then incubated in 1: 10,000 anti-digoxigenin coupled with an enzyme alkaline phosphatase antibody in blocking solution at 4 °C overnight. After incubation, samples were washed with PBST and transferred to staining buffer. The staining reaction contained NBT and BCIP in staining buffer until the signal was sufficiently strong. All photos were acquired by bright-field microscopy with a digital camera (Leica DFC420 C, Leica Microsystems, CH-9435 Heerbrugg, Germany), except for the co-localization of mRNA and protein signals of GHRL.

Immunocytochemistry

After *zghrl* mRNA hybridization, slides were washed twice in PBST followed by blocking with a solution containing 2% sheep serum and 2 mg/ml BSA in PBST for 4 h at room temperature. Samples were incubated at 4 °C overnight in primary antibody for octanoylated rat GHRL1-11 (1: 100) in blocking solution (Hosoda et al., 2000). A series of washes followed incubation with an FITC-conjugated anti-rabbit secondary antibody (1: 200) for 2 h at room temperature. Samples were washed several times with PBST and immediately examined under a fluorescence microscope (Axioplan 2 Imaging), and images were acquired by a charged-couple device (CCD) monochrome camera (AxioCam HRm, Carl Zeiss Vision, Munchen-Hallbergmoos, Germany).

Synthetic goldfish (g)GHRL peptide injection

Approximately 6-month-old zebrafish were acclimated in aquariums for a week prior to an intraperitoneal (IP) injection of octanoylated 12-amino acid peptide gGHRL (Miura et al., 2006). gGHRL stocks were made in sterile double-distilled H_2O and stored at -20 °C. Stocks were diluted in fish physiological saline solution (FPSS) for the *in vivo* study. Each tank contained 20 fish with an average body weight of 500 mg. Fish were fed 2% of the food

equivalent of their body weights daily. Two hours prior to the injection, the fish were anesthetized with 0.03% MS222. In the experimental group, fish were IP injected with 10 μL of gGHRL containing 100 ng/g of body weight based on a previous study of goldfish (Unniappan and Peter, 2004), with a similar volume of FPSS used for the control group. Lesser concentration was also used, 50 ng/g body weight of gGHRL, however, mRNA expression of target genes did not show any significant changes (data unpublished). After the injection, fish were reared in tanks for the successive time-course (15, 30, 45, and 60 min post-injection). Brains were surgically collected in anesthetized fish for each time-course, with five fish brains pooled to meet the desired quantity for total RNA isolation for each replicate (n=4) (20 fish for each time-course for a total of 80 fish). Samples were processed for total RNA and first-strand cDNA synthesis as described above and used for succeeding quantitative real-time (q)PCR.

qPCR

The qPCR analysis followed the methods and materials described in a previous study (Tseng et al., 2007). Genes of interest in the adult zebrafish brain, such as *zghs-r1a*, *zghs-r2a, zinsa*, *zins-r1*, *zins-r2*, *zgcgb1*, *zgcgb2*, *zgcg-r1*, *zgcg-r2*, *zgpb*, *zgpl*, *zgpm1*, *zgpm2*, *zgsm*, *zglut1a*, *zglut1b*, *zglut8,* and *zglut13.1,* were analyzed. All values were computed using a standard curve and normalized to *zβ-actin* which served as the internal positive control. Primer sets used for the qPCR are listed in **Table 2**.

Glycogen content analysis

The glycogen content was determined as described in a previous study (Chang et al., 2007). Brains of at least three adult zebrafish were pooled together for each replicate (n=6), homogenized in 30% KOH, and incubated in boiling water for 30 min. Samples were

incubated at room temperature for 20 min, then two volumes of 100% ethanol and two or three drops of Na_2SO_4 were added to precipitate the glycogen overnight. Samples were centrifuged, washed with 66% ethanol, and completely dried. The glycogen content was analyzed in 0.2% anthrone reagent dissolved in H_2SO_4 using a Synergy HT spectrophotometer (BIO-TEK, Winooski, Vermont, USA) (with excitation at 625 nm).

Statistical analysis

Values are presented as the mean ± standard deviation (S.D.). The gGHRL-injected group and the respective control group were compared using Student's *t*-test. Differences among groups were identified by one-way analysis of variance (ANOVA) (Tukey's method). A significant difference was accepted at (*) $p < 0.05$.

Results

Cloning of zghrl and zghsr cDNAs

Zebrafish preproGHRL was searched for in the NCBI nucleotide database and using BLAST in the Ensemble database. *zghrl* is located on chromosome 6 p27301754-4862 (accession nos.: **NM_001083872**, **EU908735**, and **ENSDART00000076405**). The gene organization and expression of *zghrl* were previously published (Amole and Anniappan, 2008; Kojima and Kangawa, 2005).

Two isoforms of the GHS-R were found in zebrafish, *zghs-r1a* (accession nos. **XM001335981**, **ENSDART00000078695**, and **LOC795753**) and *zghs-r2*, with additional splice variants (*zghs-r2a*, accession nos. **XM001340372**, **ENSDART00000079721**, and **LOC100000134**; *zghs-r2b*, accession no. **ENSDART00000097827**, a novel transcript). The Ensemble genome database was searched for the second splice variant.

Transcript tissue distributions of zghrl and zghsr

zghrl mRNA was ubiquitously distributed in almost all tissues analyzed (brain, eyes, gills, heart, liver, stomach, kidneys, and spleen, but not muscles) and was obviously highest in the intestines (**Figure 2A**). The two major isoforms of the zGHRL receptors, *zghs-r1a* and *zghs-r2a,* were both expressed in the brain (**Figure 2A**). Aside from brain expression, both receptors were also present in other tissues with very minimal expression levels (*zghs-r1a* was in the eyes, and *zghs-r2a* was in the eyes, gills, heart, liver, intestines, skin, and muscle). Other genes of interest, including *zins-r1* and *zins-r2,* were present in the brain (**Figure 2A**), as well as *zgpb*, *zgpl*, *zgpm1*, *zgpm2,* and *zgsm* (**Figure 3**), with *zgpb* and *zgpm2* showing the strongest transcript expressions. In addition, the *zgsl* transcript was present in the eyes, liver, and spleen, but was not found in the brain (**Figure 3**).

GHRL and GHS-R mRNA (zghrl and zghsr) expression and protein localization in the adult zebrafish brain

Cryosections of adult zebrafish brains illustrated detailed *zghrl* mRNA expression in the hypothalamus and preoptic lobe region (**Figure 1A-C, J**). The protein expression of zGHRL showed co-localization with *zghrl* mRNA signals (**Figure 1K-M**). Zebrafish GHS-Rs, *zghs-r1a* (**Figure 1D-F**) and *zghs-r2a* (**Figure 1G-I**), were both expressed in several brain regions including the hypothalamus and preoptic lobes. The brain anatomical terminology and schematic drawing used in this study are based on nomenclature of the *Zebrafish Brain Atlas* (Wulliman, et al.,1996).

Effects of exogenous gGHRL on the expression of several hormones, receptors, enzymes, and transporters in the brain

Effects of the GHRL-GHS-R system on brain-produced INS and other important metabolic factors were determined to show the scope of GHRL's regulatory actions in the CNS. Exogenous gGHRL significantly stimulated increases in *zghs-r1a* and *zghs-r2a* transcripts in the adult zebrafish brain (**Figure 2B**, **C**, respectively). Significant upregulation was observed in the transcript level of *zghs-r1a* at as early as 15 min post-injection and continued for up to 60 min post-injection, while a transient increase in *zghs-r2a* was observed at 15 min post-injection.

With the extrapancreatic synthesis of INS, significant suppression was observed in the transcript level of *zinsa* as early as 15 min post-injection up to 60 min post-injection (**Figure 2D**), but the *zinsb* transcript could not be detected in brain templates. In addition, minimal suppression of both *zins-r1* and *zins-r2* was observed in a transient manner at 30 and 60 min post-injection (**Figure 2E** and **F**, respectively). On the contrary, significant increases in

mRNA synthesis of *zgcgb1* (**Figure 4A**) and *zgcgb2* (**Figure 4B**) were observed at 15 min post-injection; however, *zgcga* expression was not detected in brain templates. Moreover, *zgcg-r1* (**Figure 4C**) and *zgcg-r2* (**Figure 4D**) had evident transiently increased mRNA synthesis at 30 and 15 min post-injection, respectively.

For CGM, GP was examined, and *zgpb*, *zgpl*, and *zgpm2* were upregulated (**Figure 5A, B**, **D**); however, *zgpm1* was unchanged (**Figure 5C**). In the case of GS, *zgsm* remained unaltered (**Figure 5E**). The glycogen content was examined to determine the degree of increased GP in the energy reserves of the brain; however, no significant decrease was noted in the gGHRL-treated group compared to the control (**Figure 5F**). For the GLUT, *zglut1a* transcript synthesis was significantly suppressed starting from 45 min post-injection (**Figure 6A**). In the same manner, *zglut1b* transcripts were also suppressed but were observed earlier at 15 min post-injection (**Figure 6B**), while the control group transcript level remained unchanged throughout the sampling period. *zglut8*, an INS-dependent glucose transporter, showed a significant but unlikely increase at 60 min post-injection (**Figure 6C**). No significant change was observed in *zglut13.1* (**Figure 6D**).

Discussion

In the present study, we were able to demonstrate temporal and spatial localization of *zghrl* mRNA and zGHRL protein expressions, and the mRNA expressions of the receptors, *zghs-r1a* and *zghs-r2a,* in the adult zebrafish brain. These data confirmed previous study in zebrafish demonstrating fasting induced increased *zghrl* mRNA expression in the brain (Gnanapavan et al., 2002), thus supporting evidence for the tissue specificity of the GHRL-GHS-R axis in the CNS.

The presence of two GHS-R isoforms was confirmed in zebrafish, and these resembled those of channel catfish (Small et al., 2009). In the case of zebrafish, a novel transcript of a splice variant was found in zGHS-R2 (zGHS-Rb) unlike in the counterparts of GHS-R1 (GHS-R1a and GHS-R1b) identified in black sea bream (Chan and Cheng, 2004), channel catfish (Small et al., 2009), rainbow trout (Kaiya et al., 2009a), tilapia (Kaiya et al., 2009b), and mammals (Howard et al., 1996). Physiological necessity may have contributed to this difference in gene characteristics. For instance, a counter-regulatory effect between isoforms was observed *in vitro* between seabream splice variants (Chan and Cheng 2004). Hence, further studies could contribute to the knowledge of biological functions among gene isoforms and splice variants.

Previously, protein expressions of zGHS-R and zGHRL were localized in the intestines respectively using anti-rainbow trout GHS-R and anti-rat GHRL antibodies (Olsson et al., 2008). In the present study, using the same anti-rat GHRL antibody, protein signal was co-localized to few *zghrl* mRNA expressed in the zebrafish brain. Amino acid similarity between antigens of the antibody used was not questioned considering the single amino acid difference: rat GHRL, G<u>S</u>S(n-octanoyl)FLSP and zGHRL, G<u>T</u>S(n-octanoyl)FLSP (with the different amino acid underlined). Thus, the data are highly acceptable despite the scarcity of protein signals observed. The presence of *zghrl* and *zghs-r* mRNA in the brain was

noteworthy for implying that production indeed took place and was essential, and so GHRL's potential autocrine and paracrine roles were proposed (Korbonits et al., 2004).

In a previous study, peripheral GHRL was found to readily cross the blood-brain barrier and be transported to the brain (Banks et al., 2002). Both central and peripheral injections of gGHRL were successfully demonstrated to affect food intake in goldfish (Matsuda et al., 2006). The effect of GHRL on food regulation is prominent, but the zebrafish were not fed after the IP injection in the present study. Therefore, the present experimental design could not examine the effect of the injected GHRL on zebrafish feeding. Instead, the present study focused on GHRL's molecular regulation regarding CGM. Nevertheless, GHRL's importance to food intake is remarkable, and should be further studied in the future.

In the present study, using a similar synthetic peptide, gGHRL, to that used in previous work (Matsuda et al., 2006; Miura et al., 2006, Unniappan and Peter, 2004), the zINS transcript level in the brain was highly affected shortly after the injection. The high homology between the mature protein structures of zGHRL and gGHRL (two amino acid differences within 19-amino acid residues) could have paved the way for an efficient cross-reaction of synthetic gGHRL acting on zGHS-Rs. This effect of gGHRL on zINS synthesis could be an indication of a pathogenetic role. For instance, INS secretion was inhibited by acute GHRL administration with evident inefficient glucose consumption in gastrectomized human patients (Damjanovic et al., 2006) and normal healthy patients (Broglio et al., 2004). On the contrary, a conflicting report in rodents showed GHRL's stimulatory effect on INS synthesis (Adeghate and Ponery, 2002). These differences in GHRL's mechanism of metabolic regulation could be due to genetic heterogeneity influencing phenotypic expression among populations as suggested by Ukkola et al. (2002). In light of the situation in fish, GHRL's physiological role may vary from species to species as proposed by Amole and Unniappan (2008).

zINS transcript synthesis in brain tissues is undoubtedly present based on our data. This result coincides with previous work done in zebrafish (Papasani et al., 2006). However, unlike the previous study, insulin-b was not detected in the present study; instead, insulin-a, named *zinsa* in our study, was detected. This difference may have been due to the technique applied or the stage of the zebrafish. While they used *in situ* hybridization on zebrafish embryos, our work was accomplished using qPCR on adult zebrafish.

GCG, known to be exclusively produced in the pancreas, imparts its endocrine action on its receptors in other tissues. Fortunately, using qPCR, the presence of the zGCG ligand and receptor was established in the brain. The apparent increased zGCG expression indicates a regulatory role for zGHRL in the brain similar to the homologous role of GHRL in rodent pancreatic tissue (Adeghate and Parvez, 2002). It was suggested that GHRL only evokes its effect mainly on INS-secreting cells, since it is more involved in weight gain and growth (Adeghate and Parvez, 2002). So far, we presumed that the significant inhibition of brain INS synthesis could have signaled GCG's increased local transcript synthesis.

Glucose-associated metabolic disorders and enzymes included in the glycolytic and gluconeogenic pathways are expected to play a critical role in INS-related pathology (Bell et al., 1996). In the present study, exogenous gGHRL's action in the brain obviously altered normal intracellular signaling. In terms of glycogen degradation, GP facilitates this action to produce glucose and serves as an energy substrate for metabolic processes. INS indirectly inhibits glucose degradation (Heijboer et al., 2006) through GP. Most likely, GP's action of glucose production is consistent with the low expression of the glucose production inhibitor, INS. The transient mRNA increases in zGP isoforms demonstrate a prompt glucose-sensing mechanism in the brain, indicating that the brain's metabolic homeostasis is very sensitive, and glucose levels must be highly maintained in a narrow range to supply the high demands of this tissue. On the other hand, the brain's glycogen content was not altered. Perhaps the

increase in GP transcription could have minimal local or systemic effects. In addition, we speculated that proper glucose sensing of the brain still persists, consistent with the unvaried expression of GS (*zgsm*). GS is an enzyme responsible for glycogen synthesis that is indirectly activated by INS (Bouskila et al., 2008). Moreover, the subsequent significant increase in the INS-dependent zGLUT isoform, *zglut8,* may indicate compensatory action to store glycogen. This could be an emergency tactic for an energy supply required by the brain in case of exhaustion and functional regulation similar to tilapia (Tseng and Hwang, 2008). Although, two brain isoforms of GLUT (*zglut1a* and *-1b*) examined were inhibited, it is only a normal phenomenon since INS inhibition took place, and glucose transportation by the cell was promoted by the mechanism known as INS-stimulated glucose uptake (Shulman et al., 1990). In addition, GLUT suppression reflects GHRL's negative effect on energy storage. Hence, our results suggest that GHRL contributes to the glucose-metabolic pathophysiology particularly inhibition of INS synthesis.

In conclusion, similar to its mammalian counterparts, we demonstrated that zGHRL regulates zINS transcript synthesis by mediating its actions through its receptors, zGHS-Rs, and subsequent complex metabolic processes follow that involve the metabolic factors, zGCG, zGP, zGS, and zGLUT, which participate in the proper maintenance of balanced CGM in the brain. Hence, we propose a model for GHRL's systematic regulatory control of CGM and integrate results from previous studies (**Figure 7**). In the model, the stomach abundantly produces GHRL aside from the local GHRL production in the brain. GHRL exerts its action on its receptors (GHS-Rs) present in neurons such as NPY/Agrp proteins (Chen et al., 2004; Miura et al., 2006) and affects energy homeostasis (Cowley et al., 2003). Convincing previous reports on mammals (Budd et al, 1986; Devaskar et al., 1993; Young, 1986; Zhao et al., 2002) and fish (Hrytsenko et al., 2007; Papasani et al., 2006) proved the

presence of INS production in the brain, and together with our data, support our model, while we can only rely on qPCR for the GCG data

Nevertheless, GHRL upregulates GCG while downregulating INS synthesis in the zebrafish brain, similar to mammalian studies mentioned above (Damjanovic et al.,, 2006; Broglio et al., 2001). This increase in GCG favors glycogen degradation facilitated by GP. On the other hand, the inhibition of INS will eventually cause its downstream signaling to be compromised. Several factors also participate in this process (not discussed here). For the most of our data, the proposed mechanisms are suggestive and contribute to greater venues of research. Thus, expounding the mechanism of GHRL's action on CGM in greater detail is indeed essential.

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TABLES

TABLE 1. Primers used for Reverse Transcript-PCR (RT-PCR) Chapter I and II of Part I.

gr	**NM_001020711**	*forward* TGTTGGCTGTGCTGAGGCTGGAAG *reverse* GGACTGGAGAATGGCAAAAAGCGA
mr	**NM_001100403**	*forward* CCACAGCACACTCCTGAGGTCCAC *reverse* AGTAGAGGTAACTGTCAAAATCGG
crf **(465 bp)**	**ENSDART00000038290**	*forward* CGAGACATCCCAGTATCC AA *reverse* GATGACAGTGTTGCGCTTCT
crfr1 **(722 bp)**	**XM_691254.2**	*forward* CAGCTCACCATGAATCCAGA *reverse* GACGACTGCTTGATACTGTG
crfr2 **(443 bp)**	**XM_681362.3**	*forward* GAATCGCTTACAGAGAGTGT *reverse* ACCATCCAATGAAGAGGAAG
β–actin	**NM_131031**	*forward* TCACTGAGGCTCCCCTGAAT *reverse* TTGAAGGTGGTCTCGTGGATA

TABLE 2. Primers used for qPCR analysis in Chapter I and II of Part I.

Gene	Accession number	Sequence 5'-3'
gr	**NM_001020711**	*forward* ACAGCTTCTTCCAGCCTCAG *reverse* CCGGTGTTCTCCTGTTTGAT
mr	**NM_001100403**	*forward* ACAGAGGCAACAATGATTAGAG *reverse* GTTCTCCCACAAAGAGGGT
cyp11b1	**NM_001080204**	*forward* ATCGAGAGACACGCAGACAC *reverse* ACAGACGAGGACACCATCAC
hsd11b2	**NM_212720**	*forward* TTGGTGGAGCAGTGAAGAAG *reverse* CACAGGGCACACAGTCTCTC
foxi3a	**NM_198917**	*forward* CTCTCGCTCAATGACTGCTTC *reverse* CAGCCTGAGAGTCCGACTTTC
foxi3b	**NM_198918**	*forward* CTCAAGGTCAGTCTCCGACA *reverse* GGGTTGGTTCTTTGTGGACT
atp6v1a	**NM_201135**	*forward* GAGGAACCACTGCCATTCCA *reverse* CAACCCACATAAATGATGACATCG
nhe3b	**NM_001113479**	*forward* TGCAGACAGCGCCTCTAGC *reverse*

		TGTGGCCTGTCTCTGTTTGC
gcm2	**NM_001005603**	*forward* TCCCTGTGGTTATGACTTTGCA *reverse* TGGACTTGAGCCATGAGACACT
ecac	**NM_001001849**	*forward* TCCTTTCCCATCACCCTCT *reverse* GCACTGTGGCAACTTTCGT
ncc	**NM_001045001**	*forward* GCCCCCAAGTTTTCCAGTT *reverse* TAAGCACGAAGAGGCTCCTTG
crh	**ENSDART00000038290**	*forward* CTTGATAGGCAGCAACTAGAAGACA *reverse* CTACATTCATACGGCGGTGGA
crhr1	**XM_691254.2**	*forward* TTATCCTGCGAAATGCCACC *reverse* ACAGCCTTCTCCAAACATCCAG
crhr2	**XM_681362.4**	*forward* TCTTAATGGTGAGGTTCGGTCTG *reverse* GTGGGTGATGTGGGAATGGA
mc2r	**ENSDART00000077231**	*forward* GATTATGATGATTTGCCCAGAGAACC *reverse* CAGCGTGACTCACCAGTAACACTACA
crhbp	**NM001003459.1**	forward TTTTCATCGGCGAACCTACTGA *reverse* CATCACCCAGCCATCAAATACC

β-actin	**NM_131031**	*forward* ATTGCTGACAGGATGCAGAAG *reverse* GATGGTCCAGACTCATCGTACTC

TABLE 3. Primer used for RT-PCR in *ghrelin* study.

Accession no.	Gene	Sequence 5'-3'	Product size (bp)
ENSDART00000076405	*zghrl*	*forward* TGTGTTTCTCTTTCCTTGTGTC *reverse* TTGTGAATATTGTCAATTGCTT	322
ENSDART00000078695	*zghs-r1a*	*forward* TGAGAACAACCACCAACCTG *reverse* GTCACGACTACCTTTGCCCTA	247
ENSDART00000079721	*zghs-r2a*	*forward* TTTTACTCCTCTGGACTGTGGCTCT *reverse* TTCGGTGGCTTTACATTCATTGGT	669
ENSDART00000008302	*zins-ra*	*forward* TCCAATCTACGGCATTTCACCAGC *reverse* GGAAGAAGACCCTTCCCACCCTTT	1151
ENSDART00000105823	*zins-rb*	*forward* CCGCCAGGATACTACACCTTTAAG *reverse* GTGGGACGGCACTTTCATACCTTG	1221
ENSDART00000022830	*zgpb*	*forward* ATAAACCTTGGGCTACAGAATACC *reverse* GTAATAGTGCGGTCGCTGGAGA	639
ENSDART00000023449	*zgpl*	*forward* AGGAGCCGAAATGGGTAGACAC *reverse* ATGGCAACCTTATCAGGGAAGC	367
ENSDART00000022830	*zgpm1*	*forward* GAACGACACTCACCCTGCCTTAG *reverse* AAGGAGATGAGATTACAGTAGTGG	1103

		C	
ENSDART00000101459	*zgpm2*	*forward* ACCCAGCAGCATTACTACGAAA *reverse* CCAATCATCTGCCTCCTCTACC	339
ENSDART00000008711	*zgsm*	*forward* GGCAGGTTTGGGTCTCGTCTTA *reverse* CTCCTGGATGCGTGCTTTACTC	370
ENSDART00000014007	*zgsl*	*forward* GGTGAGGGGTGGAGTGGATGGA *reverse* AATGGACGCAACGACAGAGTAA	568
NM_131031	*zβ-actin*	*forward* TCACTGAGGCTCCCCTGAAT *reverse* TTGAAGGTGGTCTCGTGGATA	275

TABLE 4. Primers used for qPCR in *ghrelin* study.

Accession no.	Gene	Sequence 5'-3'	Product size (bp)
ENSDART00000078695	*zghs-r1a*	*forward* ACTGTCTGTCTATTGCTTTTATCTCG *reverse* TTCTGCTTAACTGTCTTGTATAGTTCTA	110
ENSDART00000079721	*zghs-r2a*	*forward* TTACTCCTCTGGACTTGTGCTCTG *reverse* TTCGGTGGCTTTACATTCATTGGT	114
NM_131056.1	*zinsa*	*forward* CCACCACCATATCCACCATT *reverse* ACCAACAGGACCAACAGAGC	94
ENSDART00000008302	*zins-r1*	*forward* GAGATGGATGGCACCAGAGT *reverse* ATTGGACAGGCCTTGATACG	127
ENSDART00000105823	*zins-r2*	*forward* TGGGACTGGAACAAACACAA *reverse* ATGCGCAGGATCTCAGACTT	92
ENSDARESTT00000021893	*zgcgb1*	*forward* TGAAGAGACATTCAGAAGGCA *reverse* CGGGACTCCACTCCTCTTAG	113
NW_001879500		*forward* CTGCAGGTACGAGCTGACAT *reverse* GAGACCAGGAGAGCACAAGA	120
ENSDART00000029459	*zgcg-r1*	*forward* CCTTGGATAATTGTTCGTTACTTG *reverse*	170

		TGATGTGCTTTTAATTTGGAGATG	
ENSDART00000104639	*zgcg-r2*	*forward*	109
		CTGCTGGTTGCCATCTTATACTGC	
		reverse	
		GACGATACTCCTCCTCAATGTCCC	
ENSDART00000022830	*zgpb*	*forward*	127
		AAATGGGTTGACACTCAGGTTGTG	
		reverse	
		CCTGCAGGTTGAAATCATTAGGAG	
ENSDART00000023449	*zgpl*	*forward*	81
		CTTCCCTGATAAGGTTGCCA	
		reverse	
		TCCACAAATATCCGCATCAA	
ENSDART00000022830	*zgpm1*	*forward*	146
		AGTATGCCCGTGAGATCTGG	
		reverse	
		ATTGCACTGGTGTTGCCATA	
ENSDART00000101459	*zgpm2*	*forward*	114
		CAAGACCAATGGCATCACAC	
		reverse	
		TTCAACTGGCTCAGATCACG	
ENSDART00000008711	*zgsm*	*forward*	111
		AGGTTTATTTTGGTCGATGGCTTAT	
		reverse	
		ATTTCTCCCACAATTCACTTTTCC	
ENSDART00000013872	*zglut1a*	*Forward*	121
		CCATTTCTCCTGGGCTTTACCTTTA	
		reverse	
		CAGATTTGGCTTTGCTTTCCTCGTT	
ENSDART00000025414	*zglut1b*	*forward*	152
		TGACCGGCCCATACGTTTTC	
		reverse	
		ATCATCTCGGTTATATTTATCTGCC	
ENSDART00000019648	*zglut8*	*forward*	189
		CATTTTGTCTGGTGTCGTCATGT	
		reverse	

		CCTGCAATGAAAAAGCCCAT	
ENSDART00000019487	*zglut13.1*	*forward*	106
		AAAGCGTGACCATGAACTCC	
		reverse	
		AACCACTCCGGTGTCATAGC	
NM_131031	*zβ-actin*	*forward*	200
		CACCTTCCAGCAGATGTGGA	
		reverse	
		AAAAGCCATGCCAATGTTGTC	

Summary of the study

In any case whether pertaining to fish or mammals, the role of endocrine control is critical for adaptation in the course of environmental change, physiological response and development. Using zebrafish as the model system we elucidated essential and highly important hormones novel physiological functions contributing essential knowledge to the field of fisheries science. The present study demonstrates the following new discoveries:

1. Exogenous cortisol significantly boosts the specification and differentiation of epidermal ionocyte progenitor cells, evident with increased density in ionocytes (NaRCs and HRCs) density.
2. Cortisol is only if not mainly mediated by GR in influencing epidermal ionocyte progenitor development via stimulating the regulatory roles of *foxi3a/3b,* the major transcription factors responsible for ionocyte progenitor specification and differentiation.
3. The epidermal cell apoptosis was also delayed upon cortisol treatment and could be possibly an additive effect on the increased ionocyte density.
4. The higher signalling factors, CRF-CRFR1 axis and related proteins are engage to skin development beginning at the epidermal stem cell. This greatly influences the succeeding events in skin epidermal ionocyte progenitor to undergo normal development.

5. The role of CRF-CRFR1 was shown to be locally present in the skin and could function as separate entity from that of cortisol-GR axis, and together completing the closed-loop cascades of signals either locally or systematically.
6. Ghrelin a neuropeptide hormone regulates insulin synthesis by mediating its action on GHS-R in the CNS and partly involved in carbohydrate-glycogen metabolism.

Conference attended:

2005, The 7th Indo-Pacific Fish Conference: The function of PMCA in the inner of zebrafish (Oral presentation)

2005, Taiwan-Japan International symposium on Marine Biotechnology and its application (Participant)

2006, Annual Meeting, The Fisheries Society of Taiwan: PMCA in the inner ear of zebrafish (Oral presentation)

2006, 14th Symposium on recent advances in cellular and molecular biology: Role of Plasma membrane calcium ATPase (PMCA) and epithelial calcium channel (ECaC) in the inner ear development of zebrafish (*Danio rerio*), (Poster)

2006, International Conference on the Ecophysiology in Marine Organisms (Participant)

2008, Annual Meeting, The Fisheries Society of Taiwan: Ghrelin expression and its effect on major metabolic factors in the brain of zebrafish, *Danio rerio* (Poster)

2009, 16th International Congress of Comparative Endocrinology: Ghrelin-growth hormone secretagogue receptor axis inhibits insulin synthesis in the brain of adult zebrafish (Poster)

2010, Annual Meeting, The Fisheries society of Taiwan: Cortisol regulation on zebrafish epidermal ionocyte differentiation and proliferation (Poster)

Awards:

1998-2001, Scholar: Sponsored by SANTEH Feeds Corp. Philippines.

2000-2001, Best StudentThesis (College-Undergraduate Level) The development of vaccines and rapid techniques in the identification of bacterial pathogens of Nile Tilapia. Central Luzon State University, Munoz, Nueva Ecija, Philippines.

2001, Recognition Award, The development of vaccines and rapid techniques in the identification of bacterial pathogens of Nile Tilapia. SANTEH Feeds Corp., Philippines.

2005-2010, Scholar: Scholarship of Foreign students awarded by National Taiwan University and/or Ministry of Education, Taiwan, ROC.

2004, 九十三學年度謝德貴、馮頌彥先生紀念獎學金論文壁報比賽優秀論文獎

2004, 九十三學年度漁科所學術論文壁報比賽佳作

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2009, Certificate of Travel award. International Congress of Comparative Endocrinology. Hongkong, SAR

2011, Certificate of Travel award. 13th Society of Chinese Bioscientist in America International Symposium, Guangzhou China PROC

Publications:

Cruz S., Shiao J.C., Liao B.K., Huang C.J., Hwang P.P. (2009) Plasma membrane calcium ATPase required for semicircular canal formation and otolith growth in the zebrafish inner ear. *J Exp Biol* 212:639-647.

Cruz S.A., Tseng Y.C., Kaiya H., Hwang P.P. (2010). Ghrelin affects carbohydrate-glycogen metabolism via insulin inhibition and glucagon stimulation in the zebrafish (Danio rerio) brain. *Comp Biochem Physiol Part A* 156:190-200.

Lee Y.C., Yan J.J., Cruz S.A., Horng J.L., Hwang P.P., (2011) Anion exchanger 1b, but not sodium-bicarbonate cotransporter 1b, plays a role in transport functions of zebrafish H^+-ATPase-rich cells. *Am J Physiol Cell Physiol* 300:C295-307.

FIGURES

PART I

Chapter I

Cortisol controls epidermal ionocyte differentiation and proliferation by targeting Foxi3 transcription factors in zebrafish (*Danio rerio*)

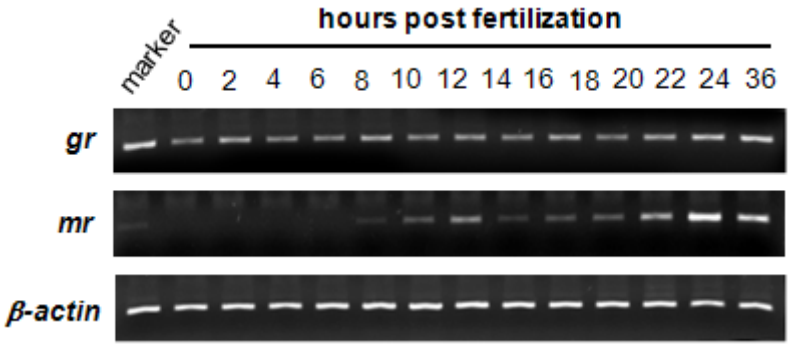

Figure (C1) 1. Genes profiles at early developmental stages. *Glucocorticoid receptor* (*gr*) and *mineralocorticoid receptor* (*mr*) mRNA expression levels. Templates starts from 0 (newly fertilized egg) hour post fertilization (hpf). *β-actin* used as positive control.

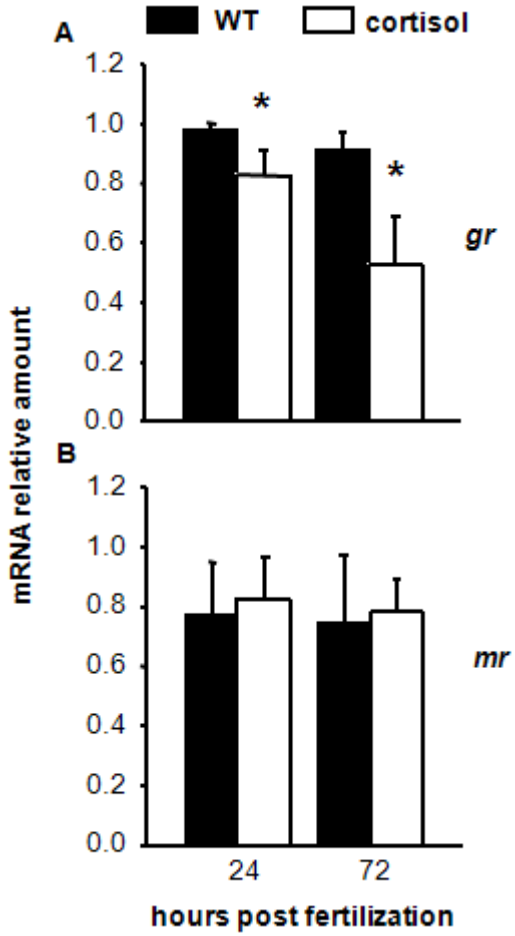

Figure (C1) 2. Corticosteroid receptor genes level of expression after cortisol treatment of zebrafish embryos. mRNA level of *glucocorticoid receptor* (*gr*) (A) and *mineralocorticoid receptor* (*mr*) (B) after cortisol treatment (20mg/L) at different sampling period. All values were normalized to *β-actin* as the internal positive control and presented as the mean ±s.d. (n=4). *Indicate the significant difference (<0.05) between wild-type (WT) and cortisol-treated groups.

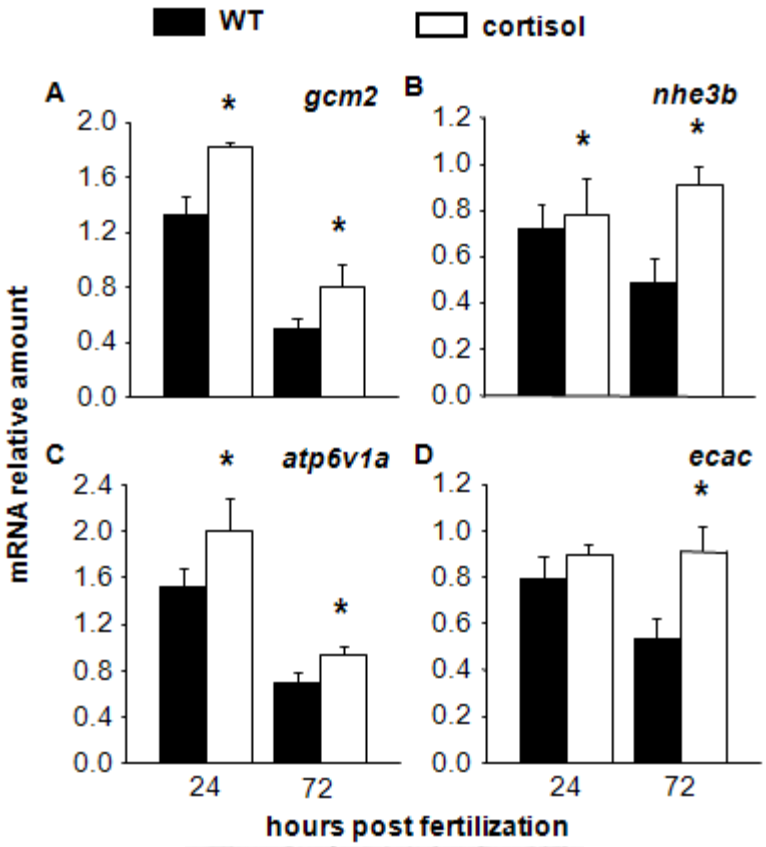

Figure (C1) 3. Ionocyte marker genes expression after exogenous cortisol treatment in zebrafish embryos. mRNA level of *glial cell missing 2* (*gcm2*) (A), *Na^+-H^+-exchanger 3b* (*nhe3b*) (B), *H^+-ATPase A-subunit* (*atp6v1a*) (C) for HRCs, and *epithelial calcium channel* (*ecac*) (D) for NaRCs after cortisol (20mg/L) treatment in different treatment duration and sampling period. All values were normalized to *β-actin* as the positive internal control and presented as the mean ±s.d. (n=4). *Indicate the significant difference (<0.05) between wild-type (WT) and cortisol-treated groups.

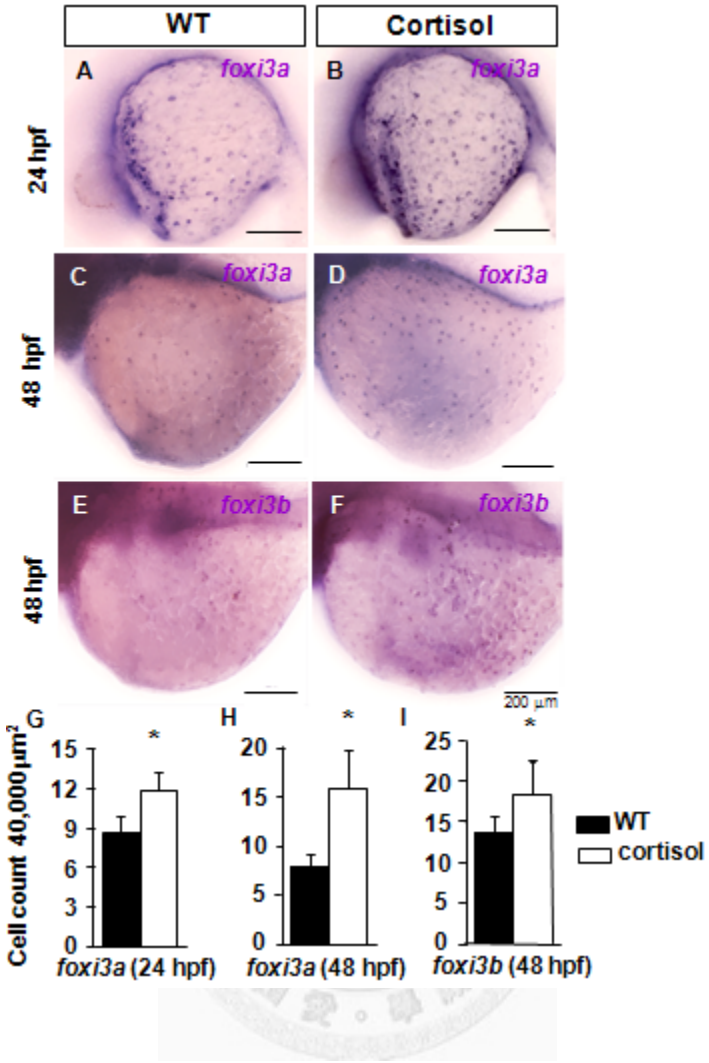

Figure (C1) 4. Foxi3 genes expression after cortisol treatment of zebrafish embryos. mRNA spatial expression of *forkhead transcription factor i3a* (*foxi3a*) (A, B) and *forkhead transcription factor i3b* (*foxi3b*) (C) after cortisol (20mg/L) treatment of different sampling periods (hours post fertilization, hpf). Statistical analysis of *foxi3a* (G, H) and *foxi3b* (I) spatial expressions. Values are presented as the mean ±s.d. (n=10-12). *Indicate the significant difference (<0.05) between wild-type (WT) and cortisol-treated group. Scale bar: 200 µm (A-J).

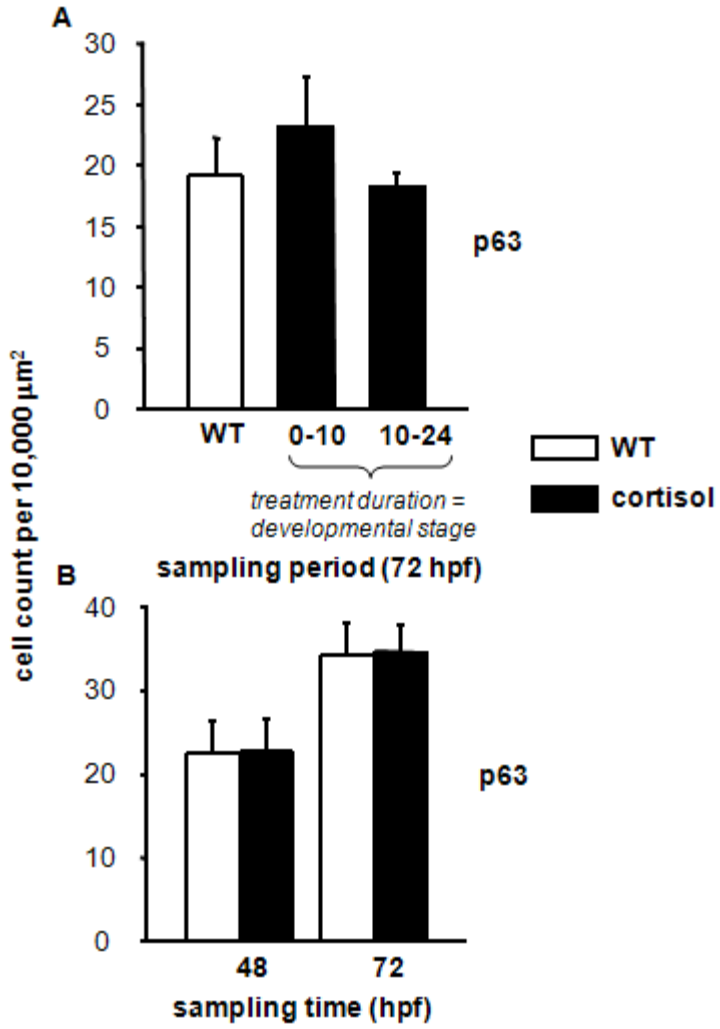

Figure (C1) 5. Epidermal stem cell number after cortisol treatment. Anti-p63 labeled the epidermal stem cell (A) in zebrafish embryos. Cortisol (20 mg/L) treatment of shorter (A) duration parallel to the embryos developmental stages (ex. 1-10 treatment; 1-10 hours post fertilization, hpf), and samples were collected at 72 hours post fertilization (hpf). Longer (B) duration of treatment collected at the end of each duration. Values are presented as the mean ±s.d. (n=10-12). No significant difference (<0.05) between wild-type (WT) and cortisol-treated groups.

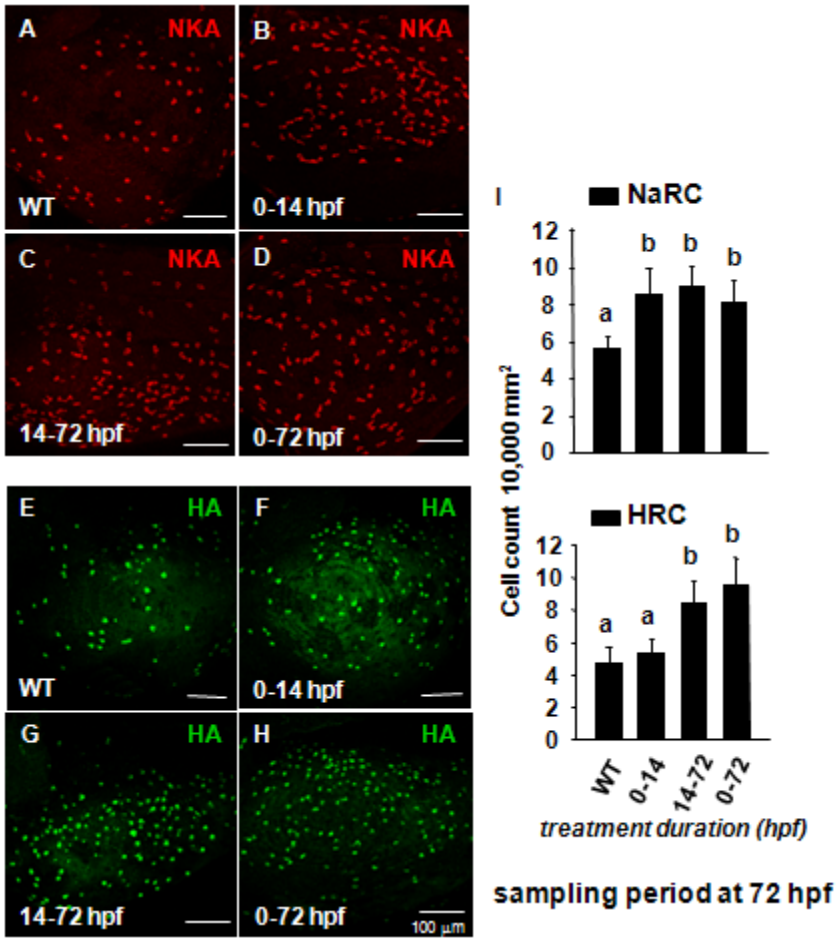

Figure (C1) 6. Ionocyte proliferation after cortisol treatment. Image representative of ionocytes, NaRCs (A-D) labeled with anti-α 5 subunit of Na^+-K^+-ATPase, and anti-H^+-ATPase for HRCs (E-H) comparing wild-type (WT) embryos with cortisol (20 mg/L) treated group. Statistical quantification of ionocytes number (I). Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate the significant difference (<0.05) between WT and cortisol-treated groups. Scale bar: 100 μm (A-H).

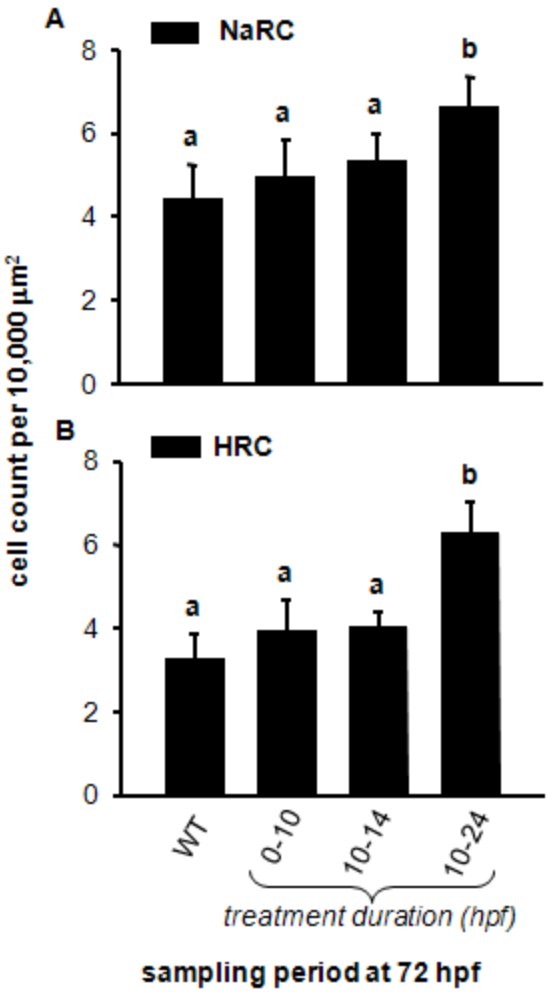

Figure (C1) 7. Ionocytes density after cortisol treatment. Ionocytes NaRCs (A) labeled with anti-α sub-unit of Na^+-K^+-ATPase and anti-H^+-ATPase for HRCs (B) of embryos treated with cortisol (20mg/L) in short duration of time are compared to that of wild-type (WT) embryos. Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate the significant difference (<0.05) between WT and cortisol-treated groups.

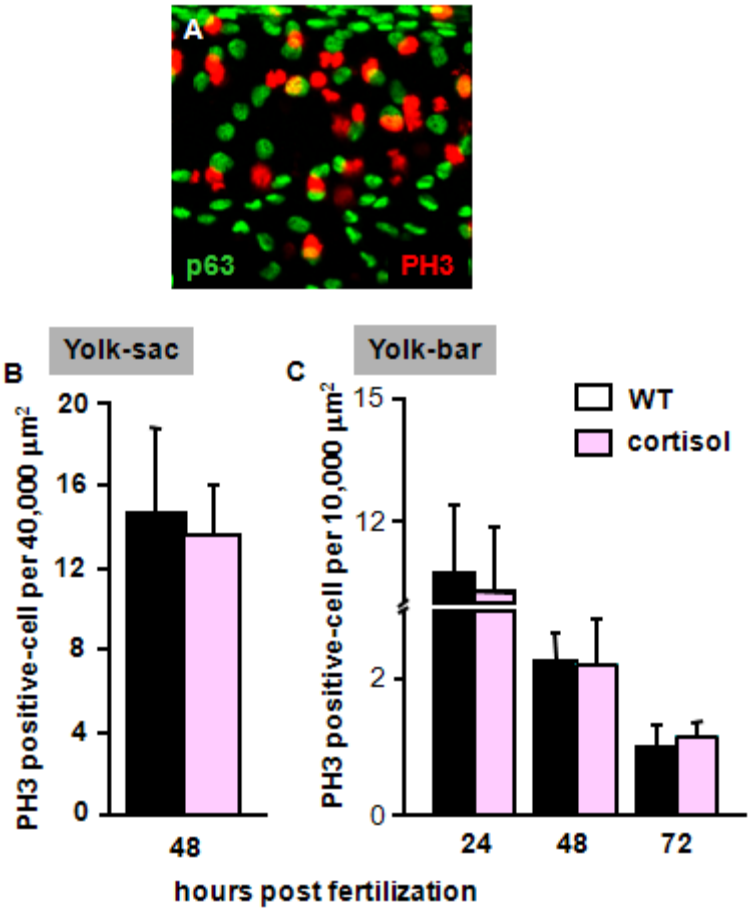

Figure (C1) 8. Cell mitosis after exogenous cortisol treatment in zebrafish embryos. Anti-phosphorylated Histone 3 (PH3) labeled dividing stem cells (A), in embryos yolk-sac (B) and yolk-bar (B) treated with cortisol (20 mg/L) collected at different time parallel to duration of treatment. Anti-p63 labeling served as reference to dividing epidermal stem cells. Values are presented as the mean ±s.d. (n=8-12). No significant difference (<0.05) between wild-type (WT) and cortisol-treated group.

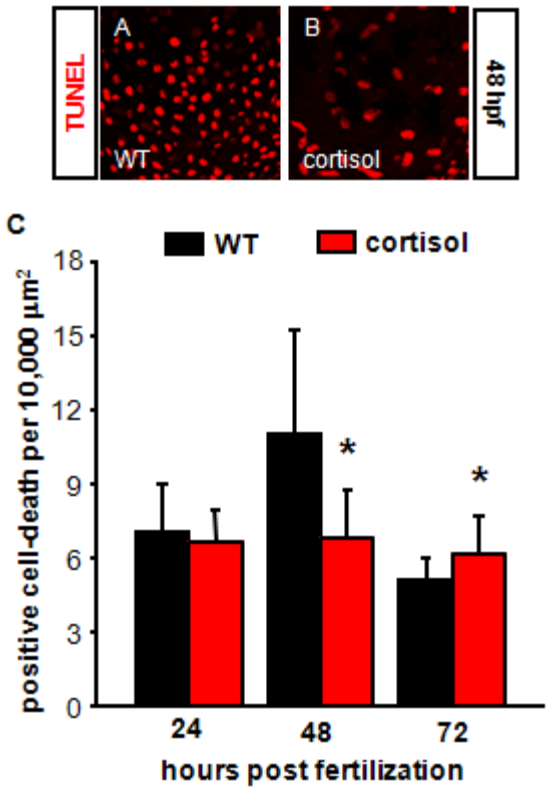

Figure (C1) 9. Epidermal cell death after exogenous cortisol treatment in zebrafish embryos. *In situ* cell-death labeled by the addition of fluorescein dUTP at DNA strand breaks by terminal transferase (TUNEL assay). Image representation for TUNEL assay of wild-tytpe (WT) (A) and cortisol (20 mg/L) group (B) sampled at 48 hours post fertilization, hpf. Cell apoptosis between WT and cortisol-treated embryos were statistically compared as collected in different period of time parallel to treatment duration (C). Values are presented as the mean ±s.d. (n=8-12). *Significant difference (<0.05) between WT and cortisol-treated group.

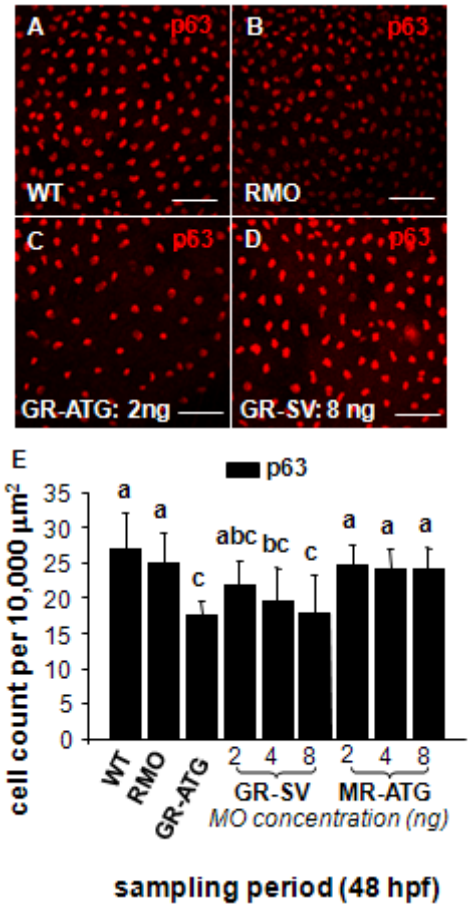

Figure (C1) 10. Epidermal stem cell number after corticosteroid receptors gene knock-down. Anti-p63 was used to label the stem cell. Image representations of yolk-sac stem cell in wild-type (WT) (A) embryos, random morpholino oligos (RMO) (B), glucocorticoid receptor-ATG MO (GR-ATG) (C) and GR-splice variant MO (GR-SV) (D) morphants. Statistical analysis of stem cell number among groups (E) were compared including mineralocorticoid receptor-ATG MO (MR-ATG) morphants. Values are presented as the mean ±s.d. (n=10-12). [abc]Indicate significant difference (<0.05) comparing WT and RMO to GR-ATG MO, GR-SV MO and MR-ATG MO groups. Scale bar:100 µm (A-D).

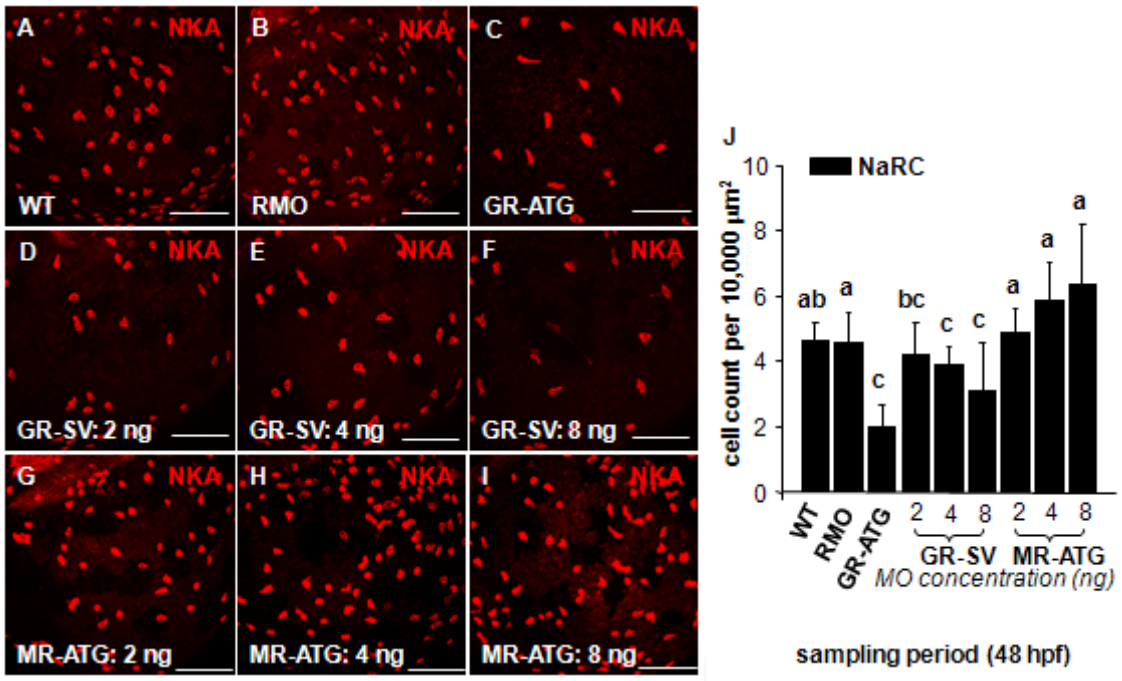

Figure (C1) 11. NaRCs density after corticosteroid receptors gene knock-down. Anti-α sub-unit of Na^+-K^+-ATPase (NKA) labeled NaRCs. Image representative of embryos yolk-sac NaRCs in wild-type (WT) (A), random morpholino oligos (RMO) (B), glucocorticoid receptor-ATG MO (GR-ATG) (C), GR-splice variant MO (GR-SV) (D, E, F) and mineralocorticoid receptor-ATG MO (MR-ATG) (G, H, I) morphants. Statistical analysis of NaRC number among groups were compared (E). Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate statistical significant difference (<0.05) among groups. Scale bar:100 µm.

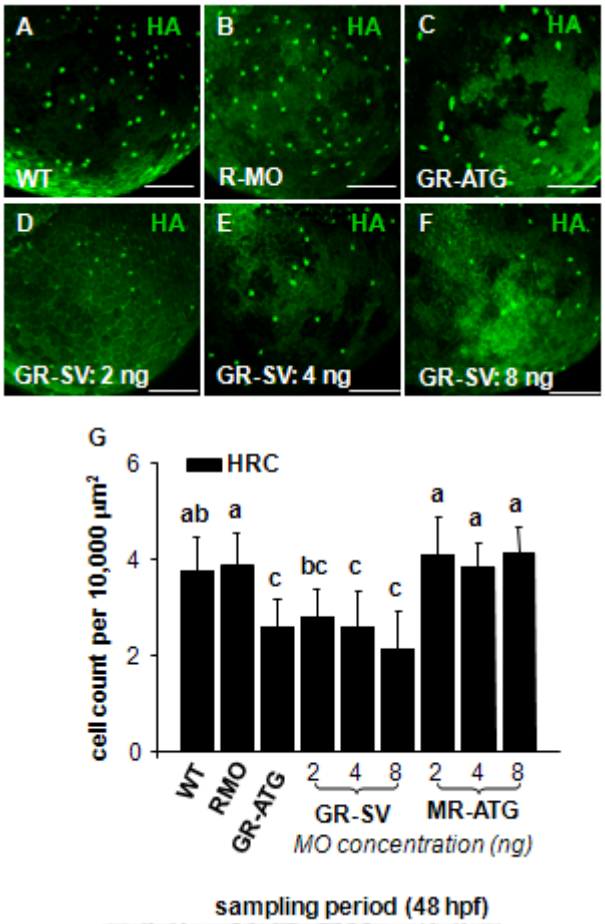

Figure (C1) 12. HRCs density after corticosteroid receptor genes knock-down. Anti-H^+-ATPase labeled HRCs. Image representative of embryos yolk-sac HRCs in embryos of wild-type (WT) (A), random morpholino oligos (RMO) (B), glucocorticoid receptor-ATG MO (GR-ATG) (C) and GR- splice variant MO (GR-SV) (D, E, F) morphants. Statistical analysis of NaRCs (G) among groups were compared including mineralocorticoid receptor-ATG MO (MR-ATG) morphants. Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate significant difference (<0.05) among groups. Scale bar:100 µm (A-F).

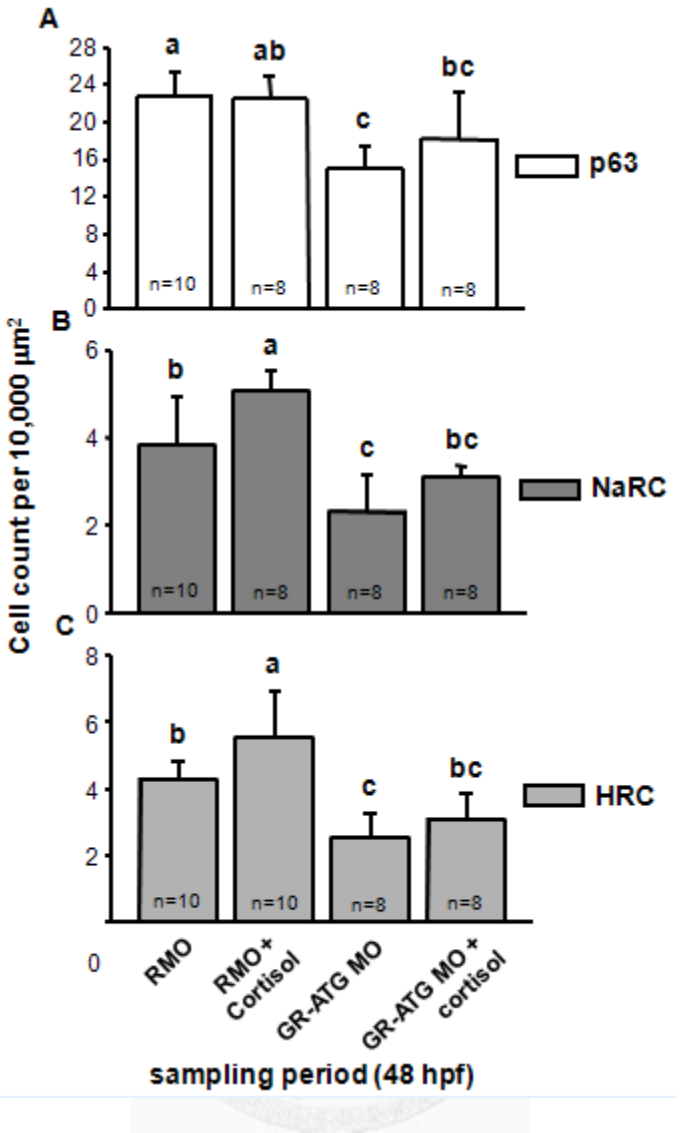

Figure (C1) 13. Stem cell and ionocytes density after glucocorticoid receptor gene knock-down with cortisol treatment. Anti-p63, anti-α sub-unit of N^+-K^+-ATPase, anti-H^+-ATPase were used to label epidermal stem cells, NaRCs and HRCs, respectively. Embryos yolk-sac epidermal stem cell (A), NaRCs (B) and HRCs (C) in random morpholino oligos (RMO) morphants were compared to glucocorticoid receptor-ATG MO (GR-ATG MO) morphants with or without cortisol (20 mg/L), respectively. Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate significant difference (<0.05) among groups.

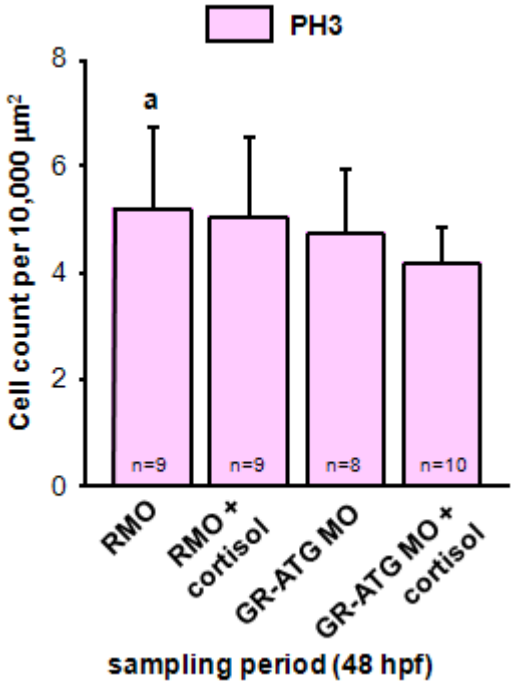

Figure (C1) 14. Cell mitosis after glucocorticoid receptor gene knock-down with cortisol treatment. Anti-phosphorylated Histone 3 (PH3) labeled dividing cells. Embryos yolk-bar with PH3 labeling in random morpholino oligos (RMO) morphants were compared to glucocorticoid receptor-ATG MO (GR-ATG MO) morphants with or without cortisol (20 mg/L), respectively. Values are presented as the mean ±s.d. (n=10-12). [a]No significant difference (<0.05) among groups.

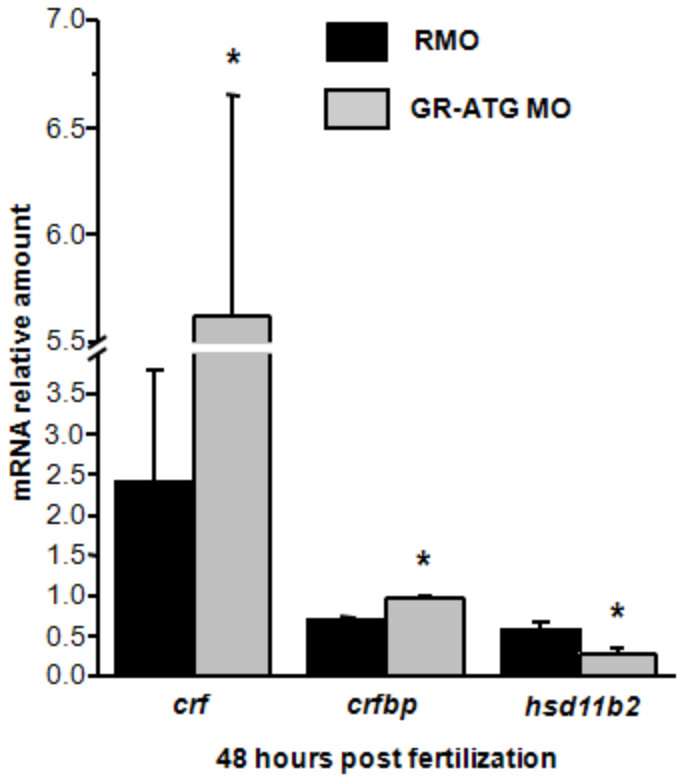

Figure (C1) 15. Related genes mRNA level after GR gene knock-down. Expression level of *corticotropin releasing factor* (*crf*), CRF binding protein (*crfbp*), and *hydroxysteroid 11-beta dehydrogenase 2* (*hsd11b2*). Templates from random morpholino oligos (RMO) morphants were compared to GR-ATG MO morphants (note: 2 ng, the highest acceptable dosage in the case of GR-ATG MO). Values are presented as the mean ±s.d. (n=4). [abc]Significant difference (<0.05) between RMO and GR-ATG MO group.

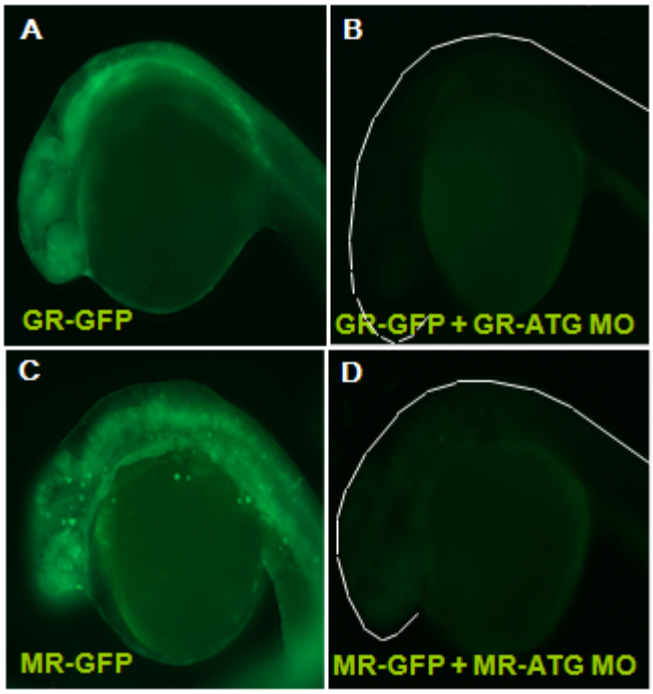

Figure (C1) 16. Morpholino oligos specificity-test. Using partial sequence spanning the target site of MOs inserted into pCS2 vector with green fluorescent protein (GFP) construct. Embryo at 24 hpf injected with *glucocorticoid receptor* partial sequence GR + pCS2-GFP alone (A), GR + pCS2-GFP with GR-ATG MO (B) and *mineralocorticoid receptor*, MR + pCS2-GFP alone (C), and MR + pCS2-GFP with MR-ATG MO (D).

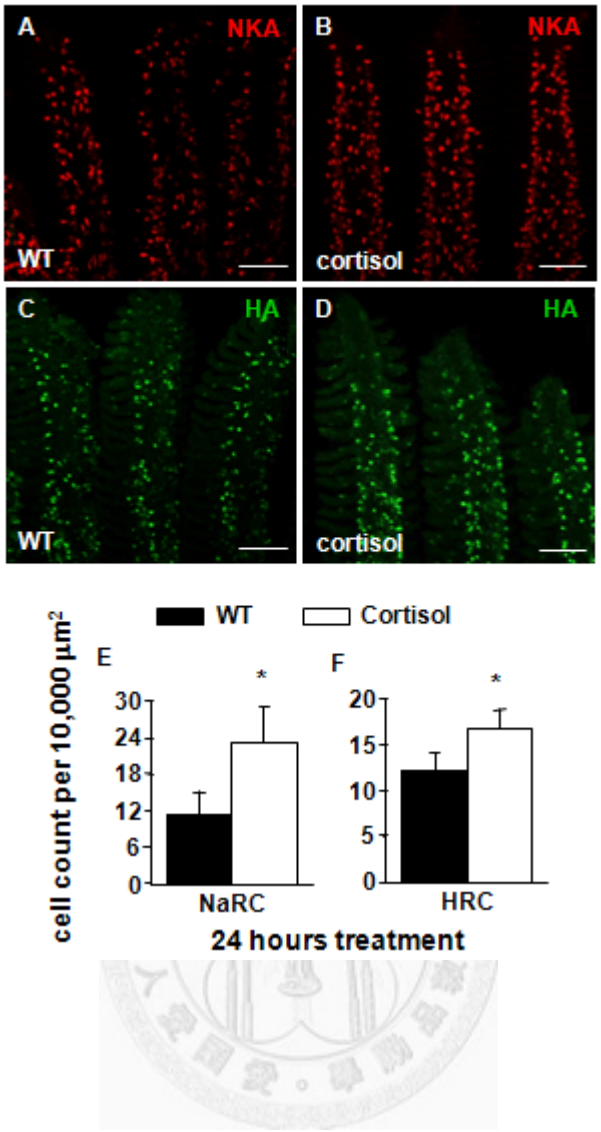

Figure (C1) 17. Ionocyte density after gill organ culture. Anti-α sub-unit of Na^+-K^+-ATPase and anti-H+-ATPase labeled NaRCs (A, B) and HRCs (C, D), respectively. Gill filaments NaRCs (E) and HRCs (F) from wild-type (WT) were compared to cortisol (20 mg/L) treated group. Values are presented as the mean ±s.d. (n=10-12). *Significant difference (<0.05) between WT and cortisol-treated group. Scale bar:100 µm (A-D).

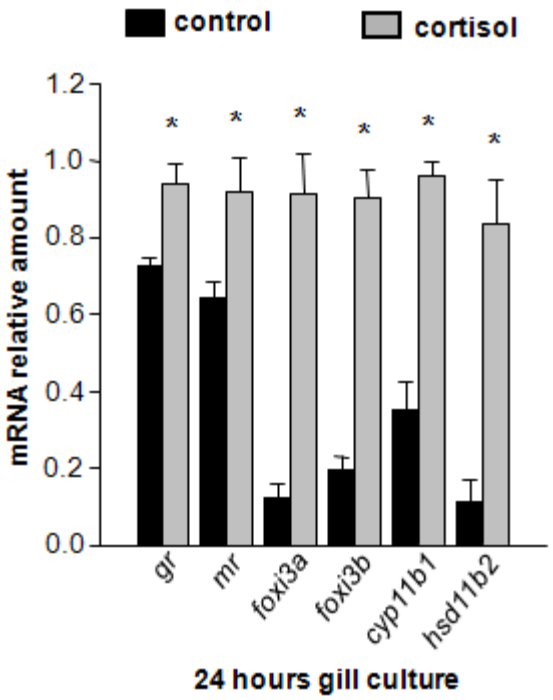

Figure (C1) 18. Exogenous cortisol effect on target genes in adult zebrafish cultured gills. mRNA expression level of *glucocorticoid receptor* (*gr*), *mineralocorticoid receptor* (*mr*), *forkhead transcription factor i3a* and *b* (*foxi3a* and *foxi3b*), *cytochrome P450, family 11, subfamily B, polypeptide 2* (*cyp11b2*), and *hydroxysteroid 11-beta dehydrogenase 2* (*hsd11b2*) after cortisol (20mg/L) treatment in 24 hours organ culture. All values were normalized to *β-actin* as the internal positive control and presented as the mean ±s.d. (n=4). *Indicate the significant difference (<0.05) between wild-type (WT) and cortisol-treated group.

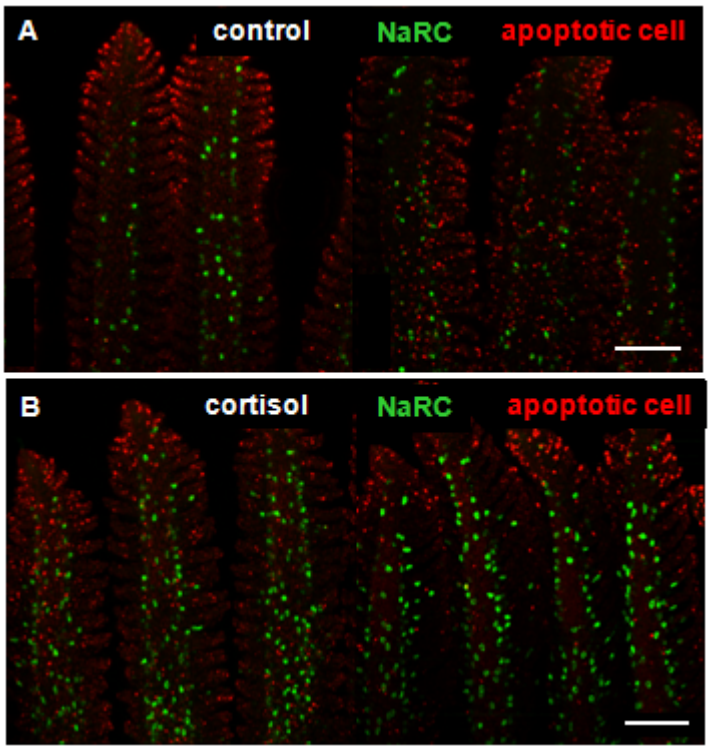

Figure (C1) 19. Cell death in gill culture with exogenous cortisol treatment. *In situ* cell-death detection by fluorescein (TUNEL assay) labeled apoptotic cells together with double-labeling of anti-α sub-unit of Na^+-K^+-ATPase for NaRCs. Control (A) in comparison to cortisol (20 mg/L) treated gills (B) for 24 hours organ culture. Scale bar:100 μm (A-B).

Chapter II

Corticotropin releasing factor (CRF), CRF-receptors and related proteins contribution on skin development of zebrafish (*Danio rerio*)

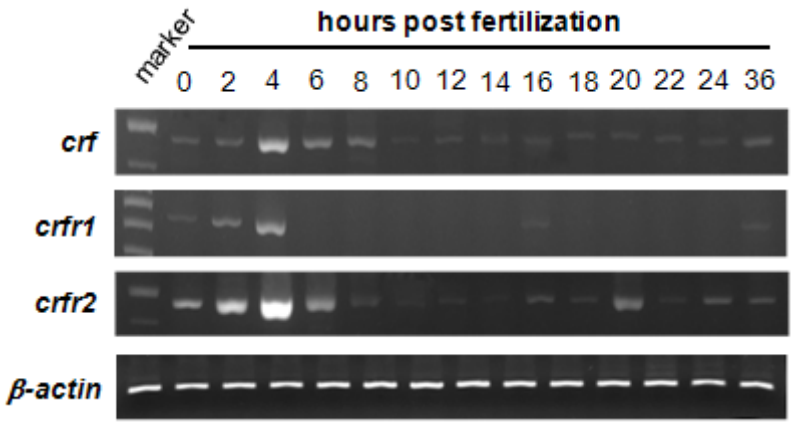

Figure (C2) 1. mRNA level of target genes at early developmental stages. *Corticotropin releasing factor* (*crf*), *corticotropin releasing factor receptor 1* and *2* (*crfr1* and *crfr2*). Templates starts from 0 (newly fertilized egg) hour post fertilization (hpf). *β-actin* used as positive control.

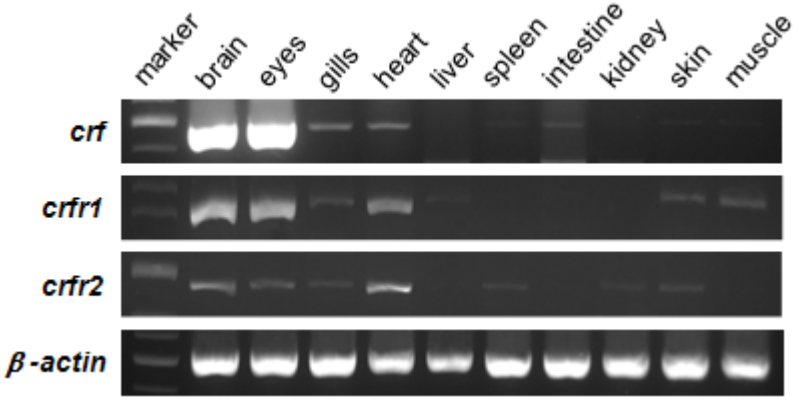

Figure (C2) 2. Tissue distribution of corticotropin releasing factor and related genes. Expression of *corticotropin releasing factor* (*crf*), *corticotropin releasing factor receptor 1* and *2* (*crfr1* and *crfr2*) in different tissues of adult zebrafish. *β–actin* serve as internal positive control.

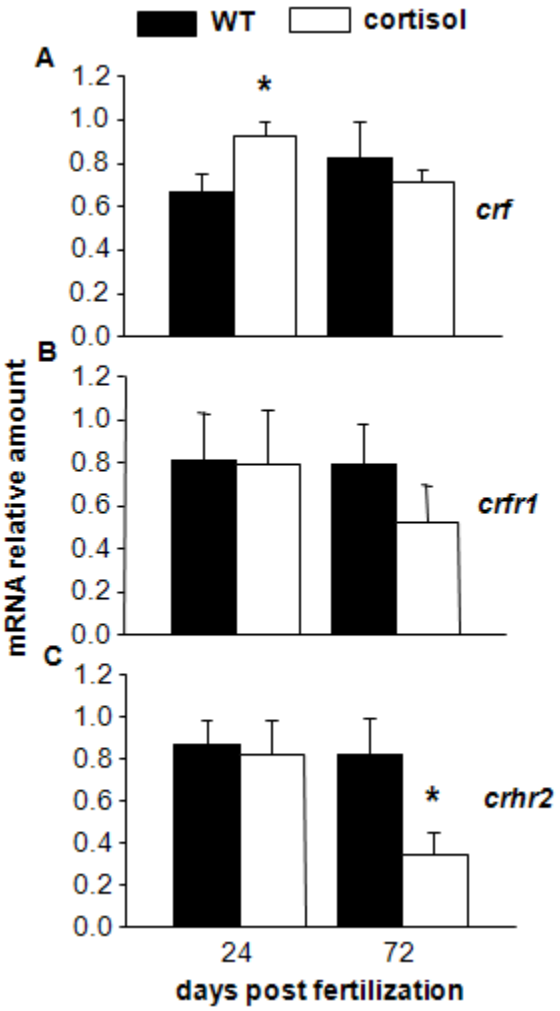

Figure (C2) 3. mRNA level of corticotropin releasing factor and receptors after cortisol treatment in zebrafish embryos. Expression level of *corticotropin releasing factor* (*crf*), *corticotropin releasing factor receptor 1* and *2* (*crfr1* and *crfr2*) after cortisol (20 mg/L) treatment. All values were normalized to *β-actin* as the internal positive control and presented as the mean ±s.d. (n=4). *Indicate the significant difference (<0.05) between wild-type (WT) and cortisol-treated group.

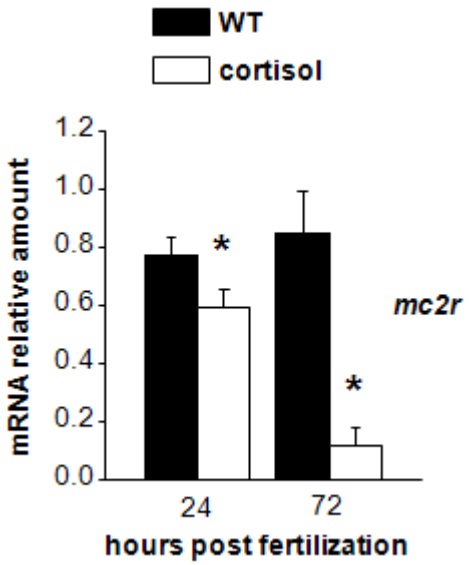

Figure (C2) 4. mRNA expression of melanocortin 2 receptor after exogenous cortisol treatment. Embryos expression level of the adrenocorticotropin releasing hormone receptor (ACTH), *melanocortin 2 receptor (mc2r)* after exogenous cortisol (20 mg/L) treatment. Samples collected at different sampling time parallel to treatment duration in hours post fertilization. Values are presented as the mean ±s.d. (n=4). *Significant difference (<0.05) between wild-type (WT) and cortisol-treated groups.

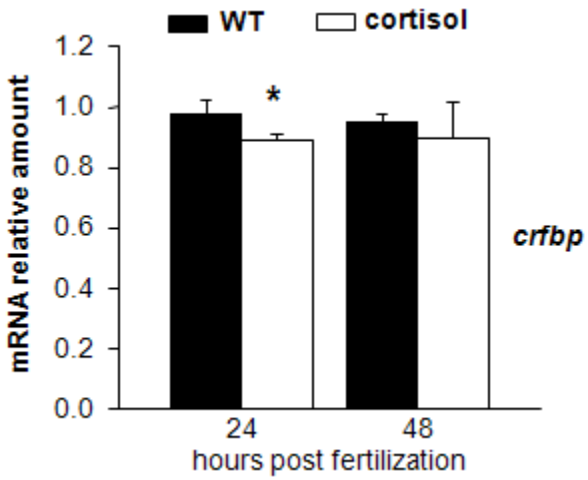

Figure (C2) 5. mRNA expression of CRF binding protein after exogenous cortisol treatment. Embryos expression level of the CRF binding protein (*crfbp*) after exogenous cortisol (20 mg/L) treatment. Samples collected at different sampling time parallel to treatment duration in hours post fertilization. Values are presented as the mean ±s.d. (n=4). *Significant difference (<0.05) between wild-type (WT) and cortisol-treated groups.

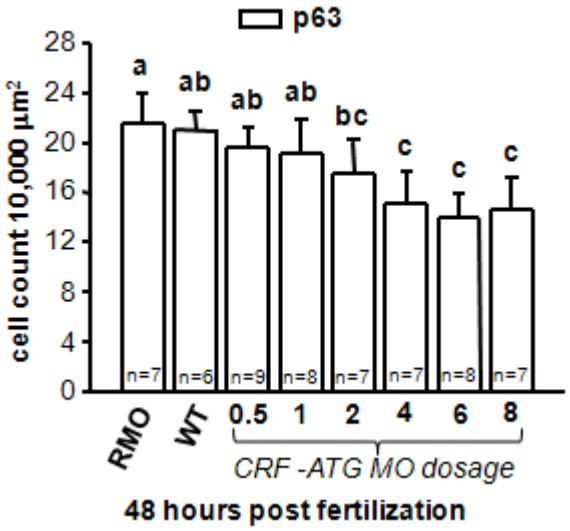

Figure (C2) 6. Stem cell number after corticotropin releasing factor gene knock-down. Anti-p63 was used to label the epidermal stem cells. Embryos yolk-sac epidermal stem cell was analyzed in wild-type (WT) embryos, random morpholino oligos (RMO) morphants compared to corticotropin releasing factor-ATG MO (CRF-ATG) morphants with increasing dosage. Values are presented as the mean ±s.d. (n=10-12). [abc]Indicate significant difference (<0.05) comparing WT and RMO to CRF-ATG MO group.

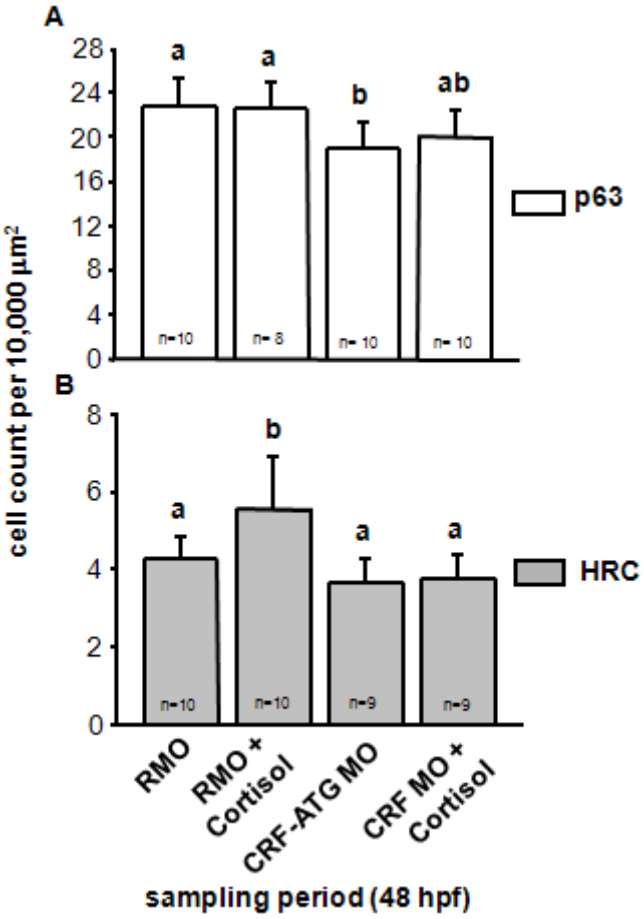

Figure (C2) 7. Effect of cortisol with corticotropin releasing factor gene knock-down. Anti-p63 and anti-H^+-ATPase labeled epidermal stem cell (A) and HRCs (B), respectively. Embryos yolk-sac epidermal stem cell was quantified in random morpholino oligos (RMO) morphants compared to corticotropin releasing factor-ATG MO (CRF-ATG MO) morphants with or without cortisol (20 mg/L) treatment sampled at 48 hours post fertilization (hpf). Values are presented as the mean ±s.d. (n=10-12). ^{abc}Indicate significant difference (<0.05) comparing RMO to CRF-ATG MO group.

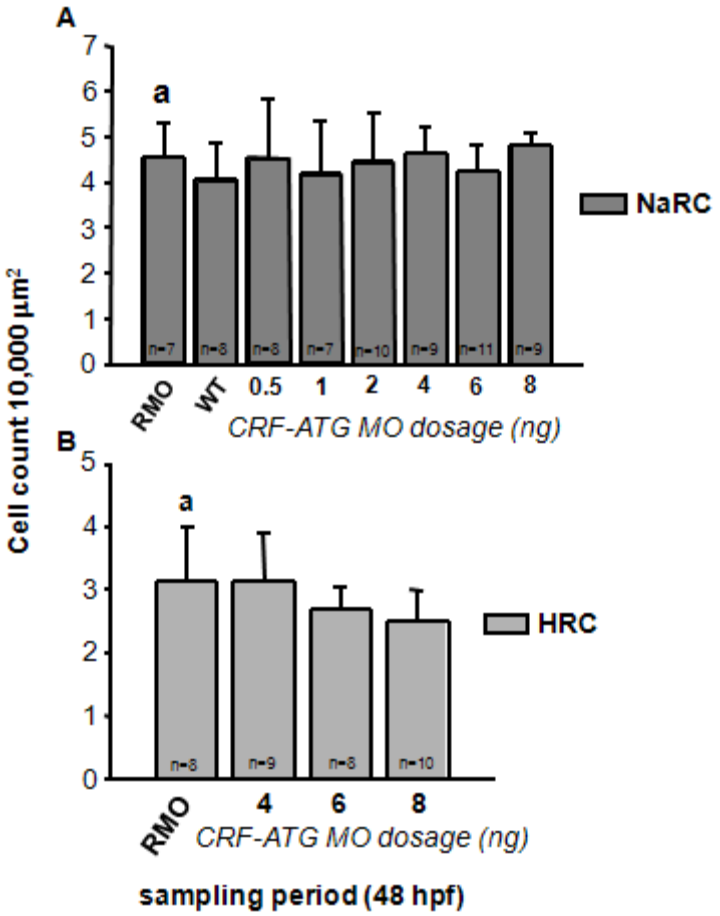

Figure (C2) 8. Effect of corticotropin releasing factor gene knock-down on ionocytes density. Anti-α subunit of Na^+-K^+-ATPase and anti-H^+-ATPase labeled NaRCs (A) and HRCs (B), respectively. Embryos yolk-sac ionocytes was quantified in random morpholino oligos (RMO) morphants compared to CRF-ATG MO morphants injected with increasing dosages. Values are presented as the mean ±s.d. (n=10-12). [a]No significant difference (<0.05) between RMO and CRF-ATG MO groups.

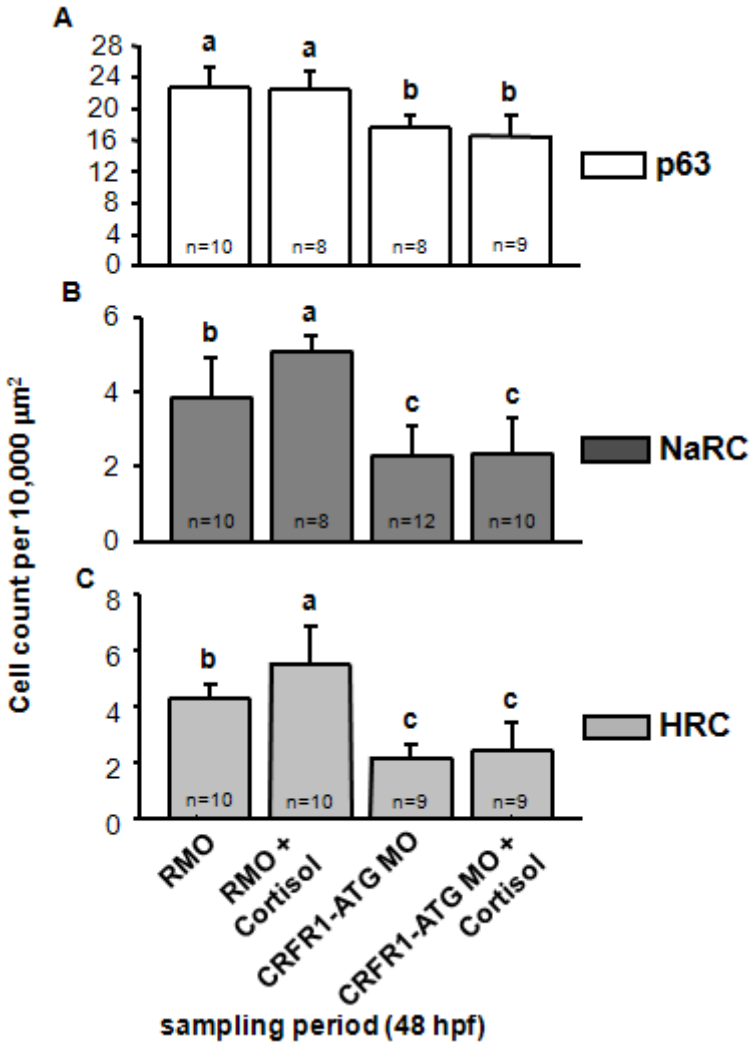

Figure (C2) 9. Effect of exogenous cortisol on ionocyte density after knock-down of corticotropin releasing receptor 1. Anti-p63, anti-α subunit of Na^+-K^+-ATPase, and anti-H^+-ATPase labeled epidermal stem cells (A), NaRCs (B), and HRCs (C), respectively. Random morpholino oligos (RMO) and corticotropin releasing receptor 1-ATG MO (CRFR1-ATG MO, 2ng) morphants with or without cortisol (20 mg/L) treatment. Values are presented as the mean ±s.d. (n=4). ^{abc}Significant difference (<0.05) between groups.

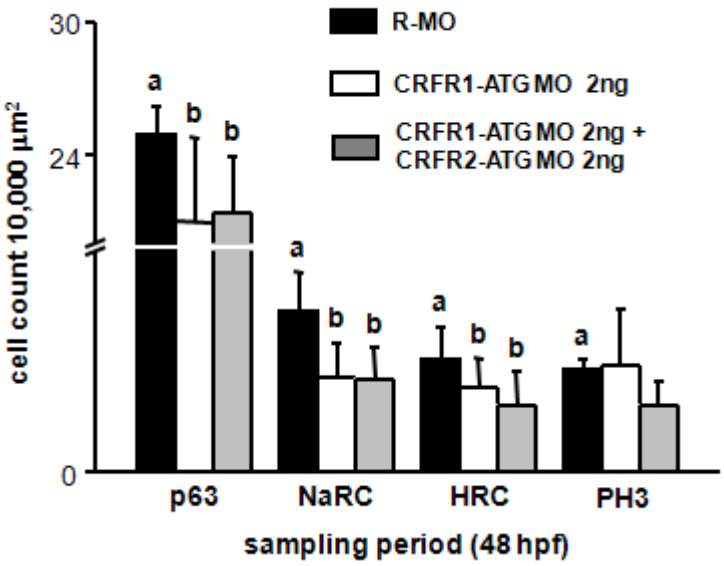

Figure (C2) 10. Effect of both corticotropin releasing factor receptor 1 and 2 genes knock-down on epidermal stem cell and ionocytes density. Embryos yolk-sac epidermal stem cell and ionocytes from random morpholino oligos (RMO) morphants were compared to morphants co-injected with CRFR1-ATG and CRFR2-ATG MOs collected at 48 hours post fertilization (hpf). Values are presented as the mean ±s.d. (n=8-12). [abc]Significant difference (<0.05) between RMO and CRFR1-ATG and CRFR2-ATG MO groups.

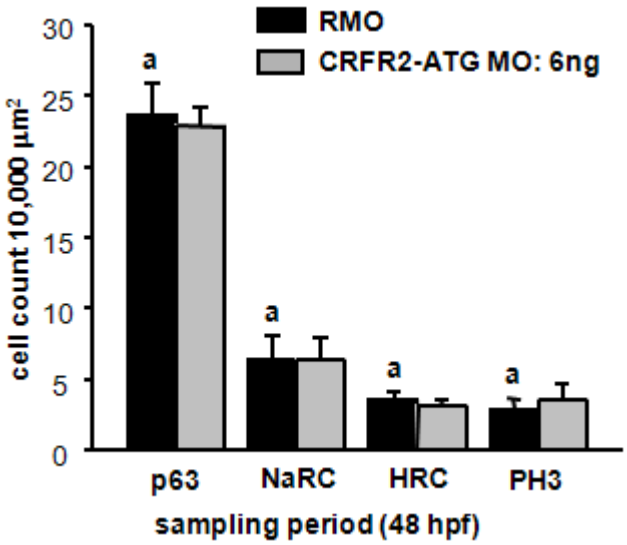

Figure (C2) 11. Stem cell and ionocytes after corticotropin releasing factor receptor 2 gene knock-down. Epidermal cell count of stem cells and ionocoytes in embryos yolk-sac from random morpholino oligos (RMO) morphants were compared to corticotropin releasing factor receptor 2-ATG MO (CRFR2-ATG MO) morphants (note: 2, 4, and 8 ng MO do not show any significant effect similar to 6 ng representing CRFR2-ATG MO data). Values are presented as the mean ±s.d. (n=8-12). [a]No significant difference (<0.05) between RMO and CRFR2-ATG MO group.

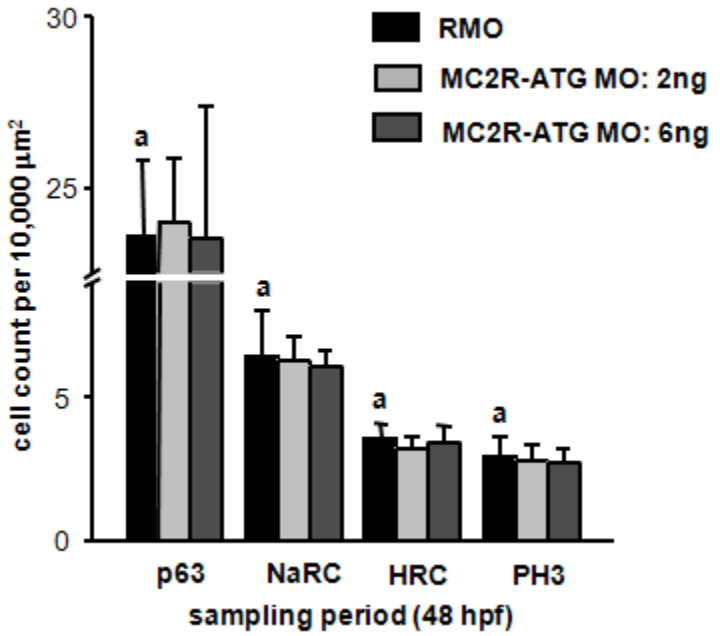

Figure (C2) 12. Epidermal stem cell and ionocytes after melanocortin 2 receptor gene knock-down. Embryos epidermal stem cell and ionocytes from random morpholino oligos (RMO) morphants were compared to MC2R-ATG MO morphants with increasing MO dosage. Samples collected at 48 hours post fertilization (hpf). Values are presented as the mean ±s.d. (n=4). [a]No significant difference (<0.05) between RMO and MC2R-ATG MO groups.

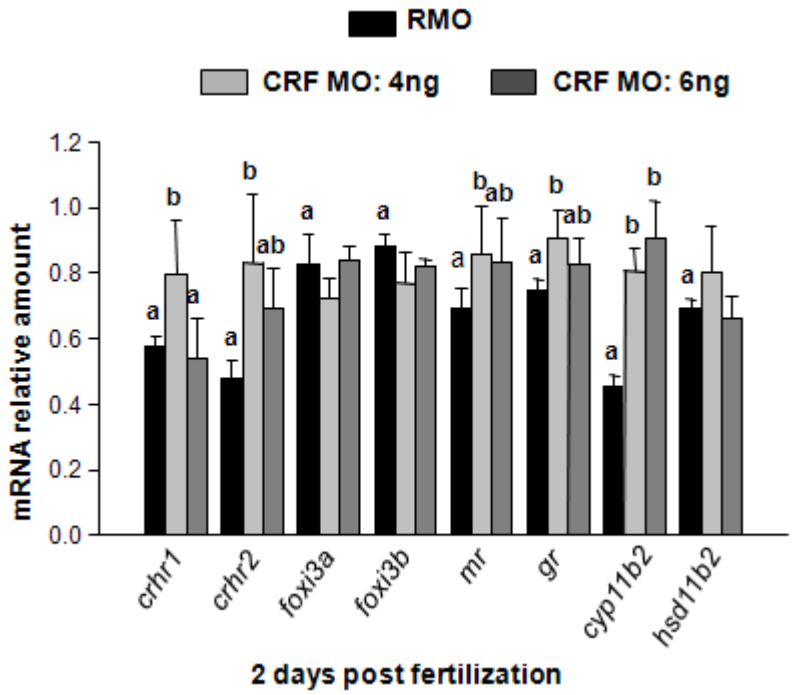

Figure (C2) 13. Target genes mRNA level after corticotropin releasing factor gene knock-down. Expression level of *corticotropin releasing factor receptor 1* and *2* (*crfr1* and *crfr2*), *forkhead transcription factor i3a* and *i3b* (*foxi3a* and *foxi3b*), *mineralocorticoid receptor* (*mr*), *glucocorticoid receptor* (*gr*), *cytochrome P450, family 11, subfamily B, polypeptide 2* (*cyp11b2*), and *hydroxysteroid 11-beta dehydrogenase 2* (*hsd11b2*). Templates from random morpholino oligos (RMO) morphants were compared to CRF-ATG MO morphants with increasing MO dosages. Values are presented as the mean ±s.d. (n=4). [abc]Significant difference (<0.05) between RMO and CRF-ATG MO groups.

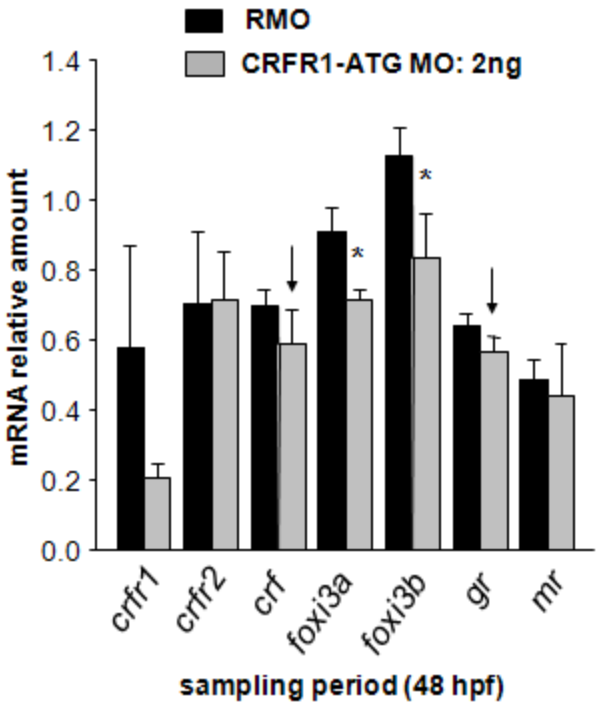

Figure (C2) 14. Target genes mRNA level after corticotropin releasing factor receptor 1 gene knock-down. Expression level of *corticotropin releasing factor receptor 1* and *2* (*crfr1* and *crfr2*), *forkhead transcription factor i3a* and i*3b* (*foxi3a* and *foxi3b*), *glucocorticoid receptor* (*gr*), and *mineralocorticoid receptor* (*mr*). Templates from random morpholino oligos (RMO) morphants were compared to CRFR1-ATG MO morphants (note: 2 ng, the highest acceptable dosage in the case of CRFR1-ATG MO). Values are presented as the mean ±s.d. (n=4). [abc]Significant difference (<0.05) between RMO and CRFR1-ATG MO group.

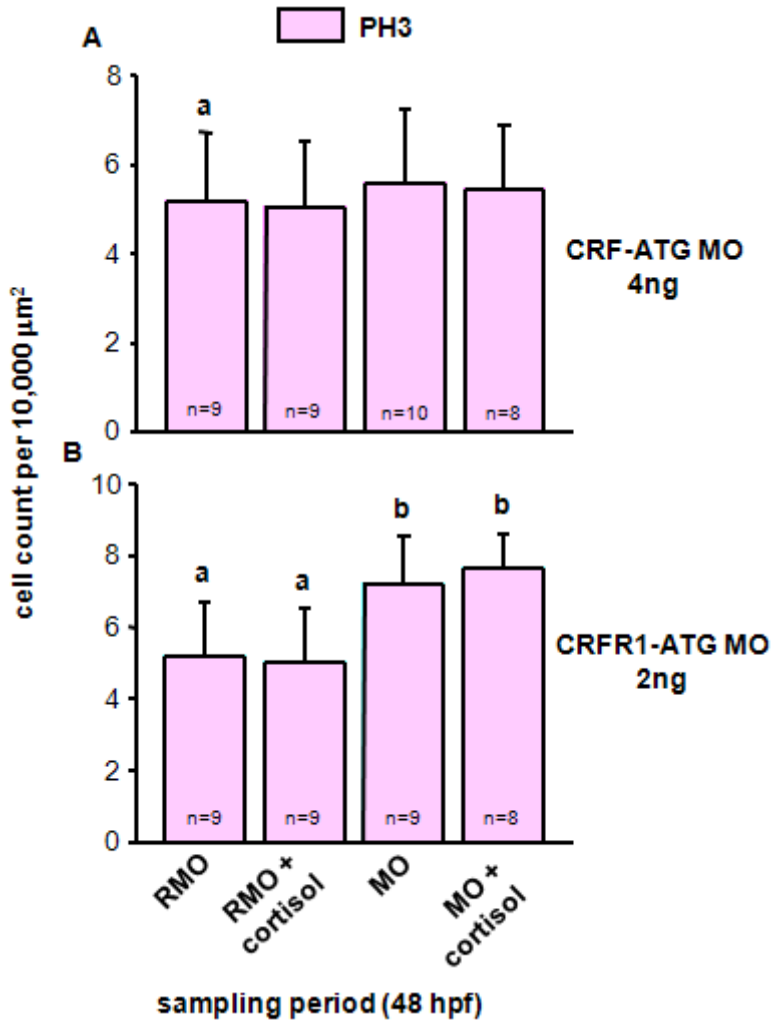

Figure (C2) 15. Cell mitosis after corticotropin releasing factor and receptor gene knock-down with cortisol treatment. Anti-phosphorylated Histone 3 (PH3) labeled dividing cells. Embryos yolk-bar with PH3-labeling in random morpholino oligos (RMO) was compared to CRF-ATG (A) and CRFR1-ATG (B) morphants with or without cortisol (20 mg/L), respectively. Values are presented as the mean ±s.d. (n=10-12). [a]No significant difference (<0.05) among groups.

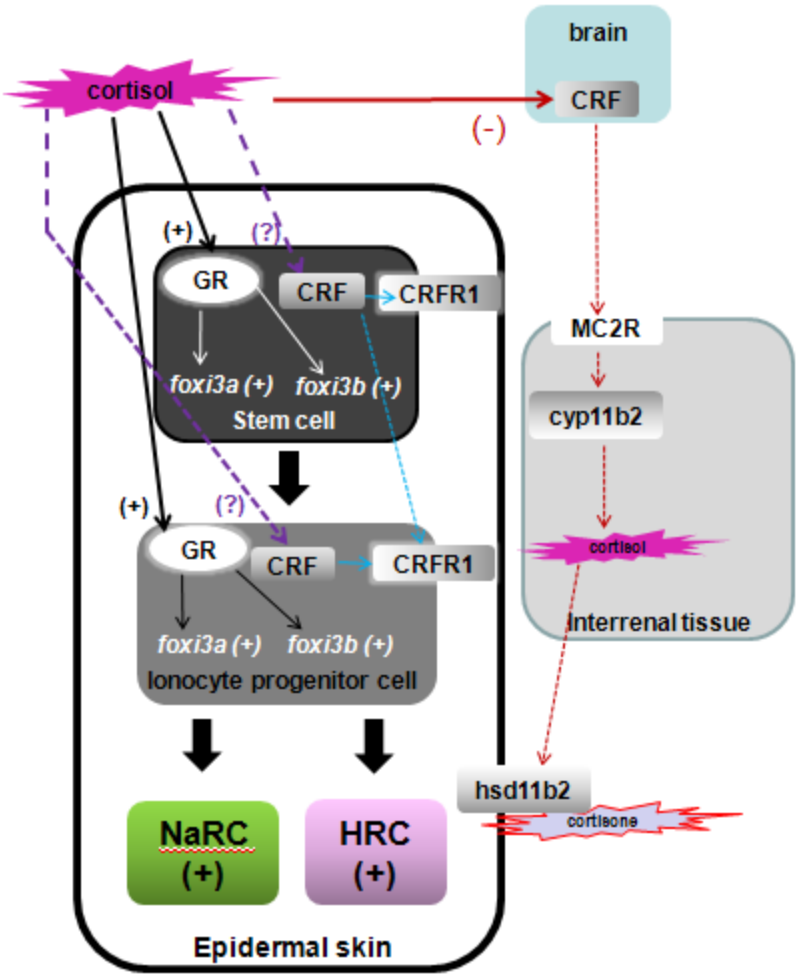

Figure (C1-2) A. Proposed model summarizing relevant findings of part I study. (*Please refer to the "Part I Conclusion" for a substantial interpretation of the model*). Black solid lines indicate cortisol action toward GR receptors down to *foxi3a/3b* with lighter weight white solid lines in epidermal stem cell and black solid lines in ionocyte progenitor cell, violet solid and dashed lines indicate cortisol action to CRF down to CRFR1 with lighter weight sky blue solid or dash lines, red solid line indicate action of cortisol to CRF in the brain down to the interrenal tissue with red lighter weight dash lines. Solid line marks solid evidence based on the study, and dash lines needs further study to clarify these data.

PART II

Chapter I

Ghrelin affects carbohydrate-glycogen metabolism via insulin inhibition and glucagon stimulation in the zebrafish (*Danio rerio*) brain

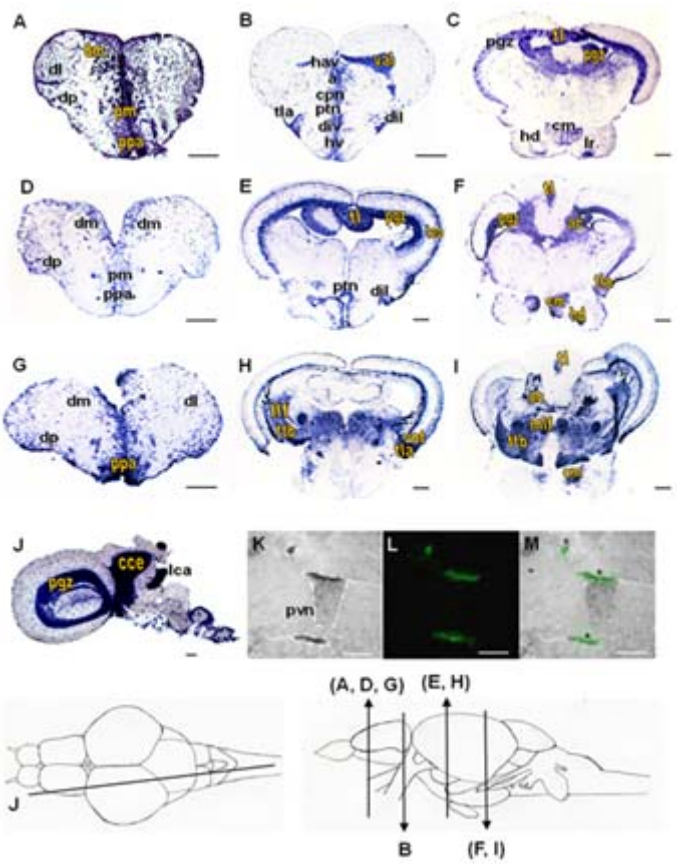

Figure 1. Spatial mRNA expressions of *zghrl, zghs-r*, and zebrafish ghrelin (zGHRL) protein localization. Adult zebrafish brain cross- (A-I and K-M) and sagittal-sections (J). Detailed *zghrl* mRNA expression along the hypothalamus and preoptic lobe region (A-C, J). *zghrl* mRNA signal (K) in the hypothalamus periventricular nucleus (PVN) co-localized (M) with the zGHRL protein signal (L). Dashed lines indicate mRNA signals, green fluorescence indicates a protein signal, and an asterisk indicates co-localization of mRNA and protein labels. *zghs-r1a* (D-F) and *zghs-r2a* (G-I) were both expressed in several brain regions including the hypothalamus and preoptic lobes. a, anterior thalamic nucleus; ac, anterior cerebellar tract; cce, corpus cerebelli; cm, corpus mamillare; cp, central posterior thalamic nucleus; d, dorsal telencephalic area; di, lateral zone of d; div, diencephalic ventricle; dm, medial zone of d; dp, medial zone of d; hav, ventral habenular nucleus; hv, ventral zone of periventricular hypothalamus; lca, lobus caudalis cerebelli; llf, lateral longitudinal fascicle; mlf, medial longitudinal fascicle; pgz, periventricular zone of optic tectum; ppa, parvocellular preoptic nucleus; pvn, periventricular nucleus; teo, tectum opticum; tla, torus lateralis; tl, torus longitudinalis; ttb, tractus tectobulbaris; val, lateral division of vulva cerebelli; vot, ventrolateral optic tract; and several caudal nuclei of the brain (unnamed). Schematic drawing shows the approximate location of the brain sections. Scale bar: 200 μm (A-J) and 100 μm (K -M).

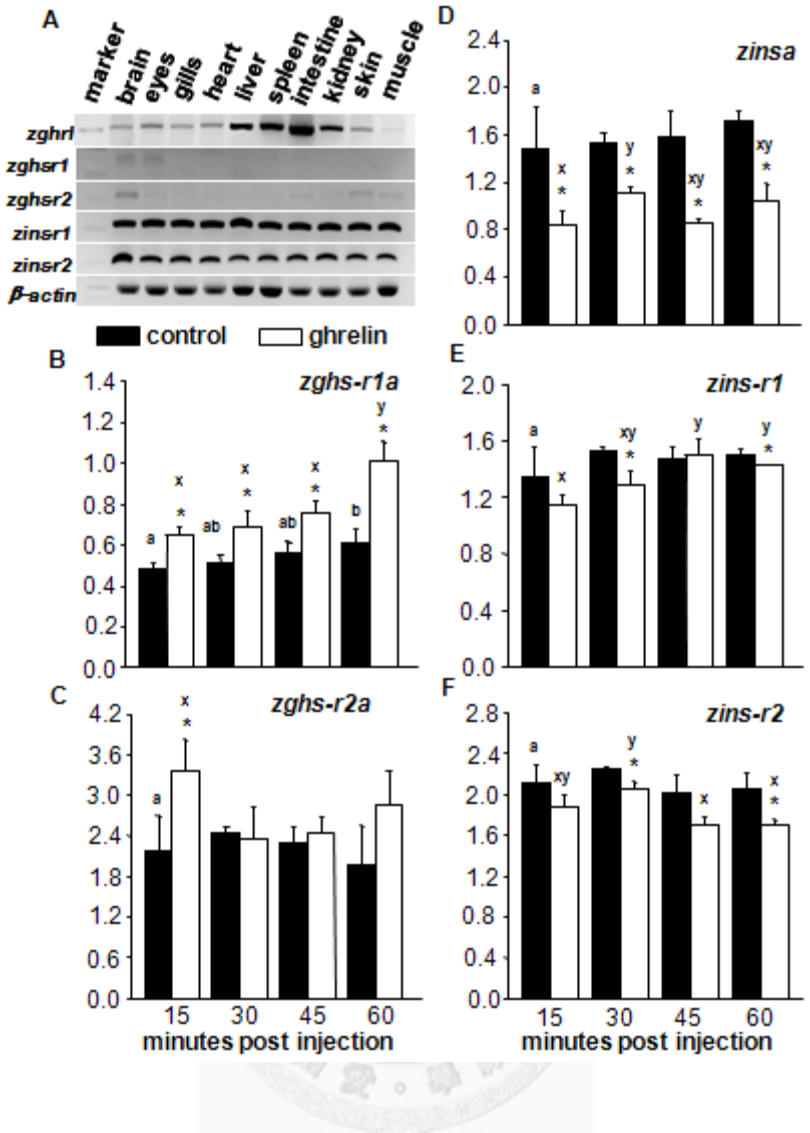

Figure 2. Tissue distribution of target genes and their expression patterns after exogenous goldfish ghrelin (gGHRL) administration using brain templates of adult zebrafish. (A) Tissue distributions of the following zebrafish genes: *zghrl, zghs-r1a*, *zghs-r2a*, *zins-r1*, *zins-r2,* and *zb-actin* which served as the positive control for the RT-PCR analysis. mRNA amounts of (B) *zghs-r1a*, (C) *zghs-r2a,* (D) *zinsa,* (E) *zinsr-1,* and (F) *zinsr-2*. All values were normalized to *zβ-actin* expression as the internal control. Values are presented as the mean ± s.d. (n=4). * Significant difference (p<0.05) between the gGHRL-treated group and the respective control group. Different letters indicate a significant difference (p<0.05) between groups.

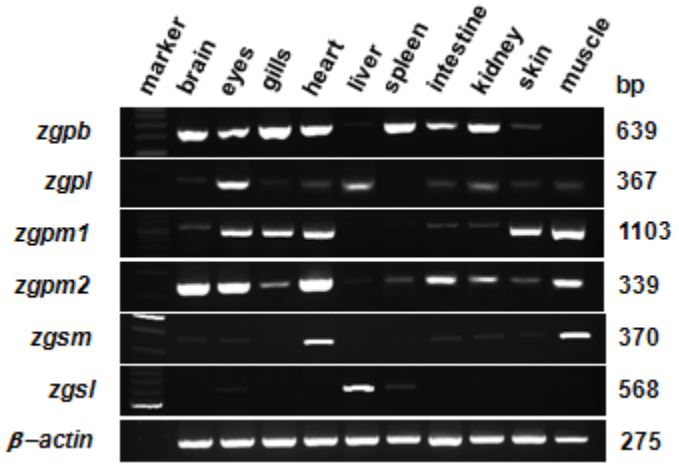

Figure 3. Tissue distribution of several genes involved in carbohydrate-metabolism. Analyzed zebrafish genes are the following: *glycogen phosphorylase brain isoform (sgpb), zgp liver isoform (zgpl), zgp muscle 1 isoform (sgpm), sgpm 2 isoform (zgpm2), glycogen synthase muscle isoform (zgsm),*and *zgs liver isoform (zgsl)*. Internal control is *β-actin*.

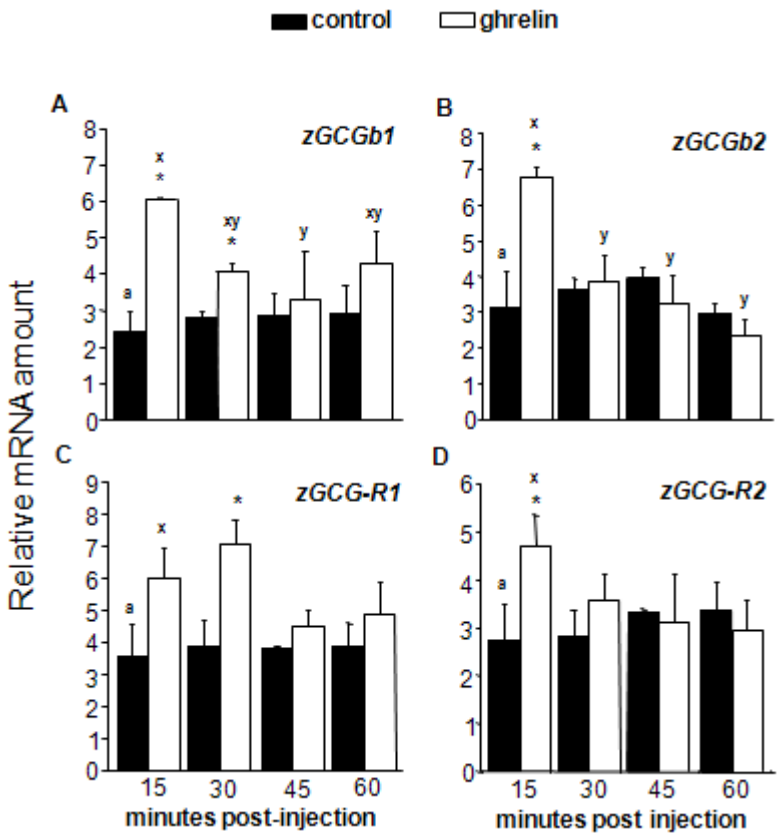

Figure 4. Expression of zebrafish glucagons (zGCGs) and their receptors (zGCG-Rs) after exogenous goldfish ghrelin (gGHRL) administration using brain templates of adult zebrafish. mRNA amounts of (A) *zgcgb1*, (B) *zgcgb2*, (C) *zgcg-r1,* and (D) *zgcg-r2*. All values were normalized to *zβ-actin* expression as the internal control. Values are presented as the mean ± s.d. (n=4). * Significant difference (p<0.05) between the gGHRL-treated group and the respective control group. Different letters indicate a significant difference (p<0.05) between groups.

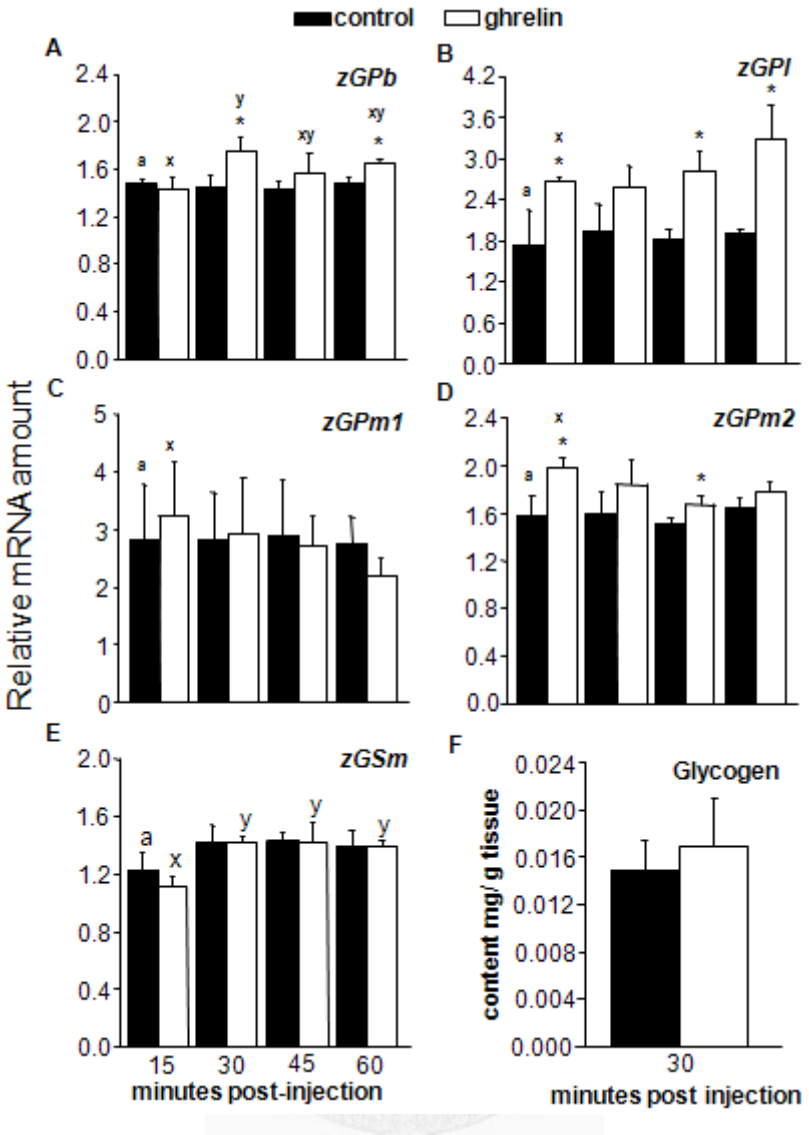

Figure 5. Expressions of zebrafish glycogen phosphorylases (zGPs) and glycogen synthase (zGS) and glycogen content after exogenous goldfish ghrelin (gGHRL) administration using brain templates of adult zebrafish. mRNA amounts of (A) *zgpb*, (B) *zgpl*, (C) *zgpm1*, (D) *zgpm2*, and (E) *zgsm*. All values were normalized to *zβ-actin* expression as the internal control. Values are presented as the mean ± s.d. (n=4). (F) Glycogen content measurement. Data are presented as the mean ± s.d. (n=6). * Significant difference (p<0.05) between the gGHRL-treated group and the respective control group. Different letters indicate a significant difference (p<0.05) between groups.

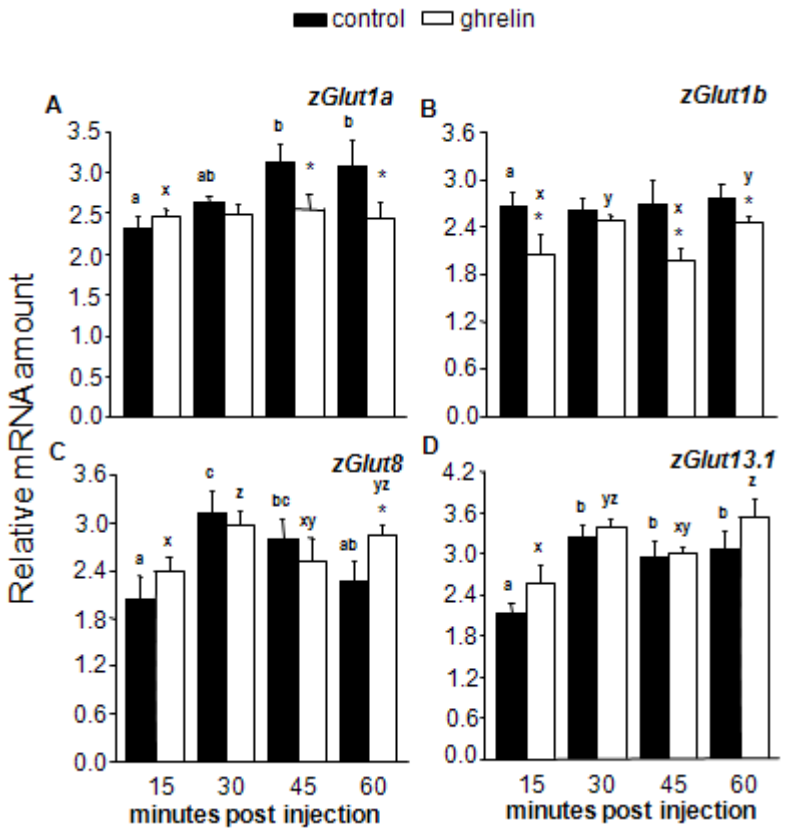

Figure 6. Expressions of zGLUTs after exogenous goldfish ghrelin (gGHRL) administration using brain templates of adult zebrafish. mRNA amount of (A) *zglut1a*, (B) *zglut1b*, (C) *zglut8* and (D) *zglut13.1*. All values were normalized to *zβ-actin* as the internal control. Values are presented as the mean ± s.d. (n=4). *Significant difference (p<0.05) between gGHRL-treated group and the control group. Different letters indicate a significant difference (P<0.05) between groups.

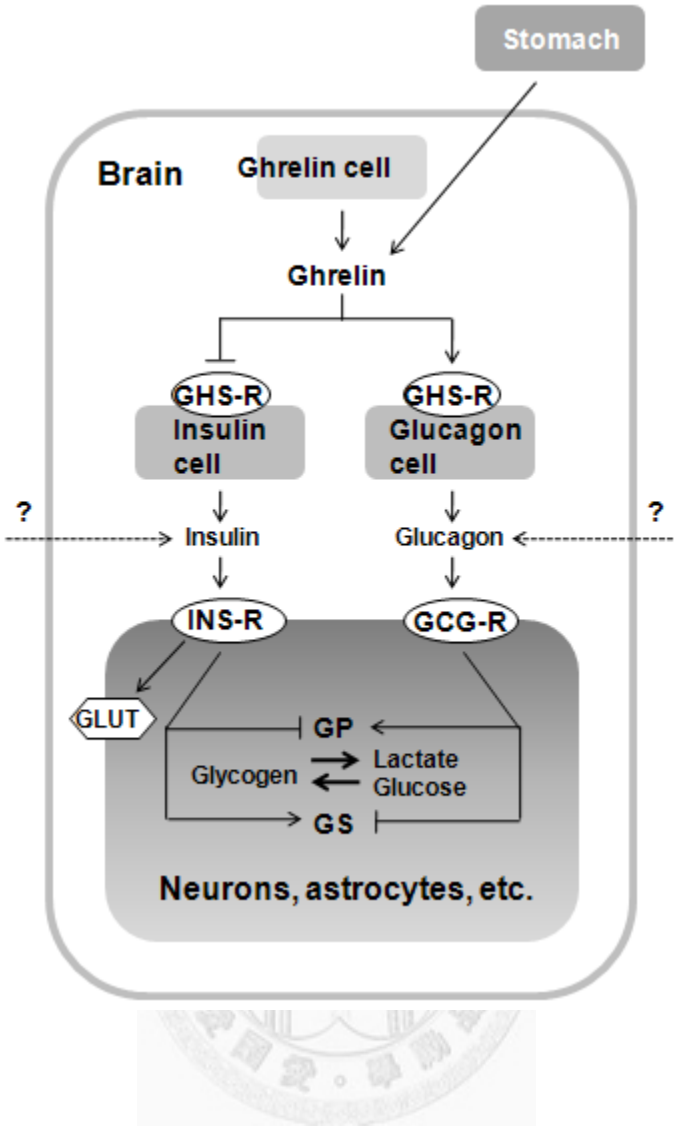

Figure 7. Ghrelin (GHRL) expression and regulatory role in brain carbohydrate glycogen metabolism. (*Please refer to the "Discussion" for a substantial interpretation of the model*). Solid lines indicate GHRL's action toward carbohydrate metabolism, and dashed lines indicate unknown mechanisms of action influencing carbohydrate glycogen metabolism.